

## Modulation of Arachidonic Acid Metabolism in the Rat Kidney by Resveratrol: Implications for Regulation of Blood Pressure

<sup>1</sup>Fawzy A. Elbarbry, <sup>2</sup>Anke Vermehren-Schmaedick and <sup>1</sup>Deepa Rao

<sup>1</sup>School of Pharmacy, Pacific University, Hillsboro, OR, USA

<sup>2</sup>Department of Physiology and Pharmacology, Oregon Health and Science University, Portland

### ABSTRACT

**Background and Objective:** The objective of this study was to investigate the effects of resveratrol (RES) on Arachidonic acid (AA) metabolism in the kidney and its effect on arterial blood pressure, using spontaneously hypertensive rats (SHR) as a model system. **Methods:** Rats were exposed to either drinking water alone (control) or RES (20 or 40 mg kg<sup>-1</sup>) added to drinking water for 7 weeks. Mean Arterial Pressure (MAP) was measured at 7-day intervals throughout the study. At the end of treatment, rats were euthanized, followed by preparation of kidney microsomes to 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 RES on kidney expression of CYP4A was also investigated by immunoblotting. **Results:** Treatment with RES resisted the progressive rise in MAP in the developing SHR in a dose-dependent manner. Consistent with these data, RES treatment led to significant reductions in both, the expression and activity of renal CYP4A isozymes, as well as the activity of soluble epoxide hydrolase (sEH). **Conclusion:** The presented studies show that RES modulates the metabolism of AA by both P450 enzymes and sEH in SHR rats, which may represent a novel mechanism by which RES protects SHR rats against the progressive rise in blood pressure.

**Key words:** Resveratrol, spontaneously hypertensive rats, arachidonic acid, metabolism, soluble epoxide hydrolase, hypertension

Pharmacologia 6 (8): 360-370, 2015

### INTRODUCTION

Hypertension is a major predictor for fatal and non-fatal cardiovascular diseases. According to the Centers for Disease Control and Prevention (CDC), it was estimated that hypertension affects more than 30% of the adults in the United States (<http://www.cdc.gov/blood-pressure/facts.htm>). Although, there is still uncertainty about the pathophysiology of hypertension, many interrelated factors have been found to contribute to persistent blood pressure elevation<sup>1</sup>. Common causes of hypertension include the following: (1) Vascular resistance, (2) Oxidative stress, (3) Endothelial dysfunction and (4) Salt sensitivity, among others. Several studies have indicated that Arachidonic Acid (AA) metabolites play a critical role in the regulation of renal vascular tone, tubuloglomerular feedback and

sodium transport<sup>2-4</sup>. Renal Cytochrome P450 (CYP)-mediated metabolism of AA produce either hydroxyeicosatetraenoic acids (HETEs; particularly, 19- and 20-HETE) or epoxyeicosatetraenoic acids (EETs, Fig. 1)<sup>2,3,5,6</sup>.

In the renal and peripheral vasculature, 20-HETE is a potent vasoconstrictor and is involved in tubule-glomerular feedback and the autoregulation of renal blood flow and glomerular filtration rate<sup>4,7</sup>. Additionally, several studies have demonstrated that the vasoconstriction effect of angiotensin II and nor epinephrine is linked in to the endogenous formation of 20-HETE in renal vascular smooth muscle and blockade of the K<sub>ca</sub> channels<sup>8,9</sup>. Moreover, infusion of inhibitors of the 20-HETE formation into the renal artery was found to attenuate the vasoconstrictor effect of these agents<sup>7,10</sup>. CYP4A11 and CYP4F2 were found to be responsible for the production of 20-HETE in the human kidney<sup>2</sup> while CYP4A1 was found to exhibit the highest catalytic activity for the formation of 20-HETE

**Corresponding Author:** Fawzy A. Elbarbry, School of Pharmacy, Pacific University, 222 SE 8th Ave., Hillsboro, OR 97123, USA  
Tel: 503-352-7356 Fax: 503-352-7270

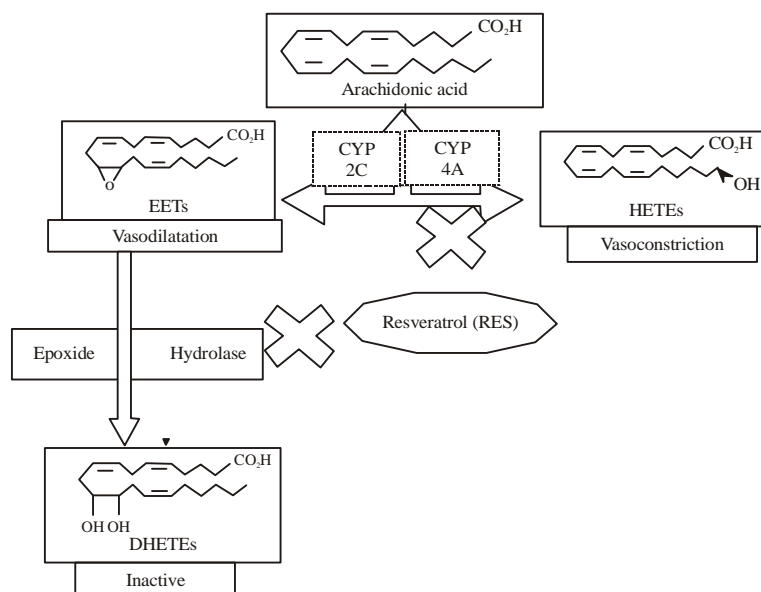


Fig. 1: Metabolism of arachidonic acid by CYP enzymes and epoxide hydrolase. The sign “x” indicates the proposed sites for the anti-hypertensive effect of RES

in the rat kidney, followed by CYP4A2 and CYP4A3<sup>11</sup>. Overwhelming evidence has established that 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<sup>3</sup>. Conversely, selective inhibition of CYP4A enzymes resulted in acute reduction in blood pressure in SHR<sup>12</sup>.

In contrast to 20-HETEs, EETs, produced by the endothelium, are potent vasodilators and considered as antihypertensive<sup>13</sup>. Several studies have led to the proposal that EETs serve as the Endothelium-Derived Hyperpolarizing Factor (EDHF)<sup>13</sup>. Deficit in EETs, especially 5,6-EET, was found to render the rat sensitive to salt-induced elevations of blood pressure<sup>14</sup>. While CYP2C8/9/18/19 are the predominant isoforms responsible for the metabolism of arachidonic acid to biologically-active EETs, CYP2C11 and CYP2C23 are responsible for most of the renal epoxigenase activity in rats<sup>3</sup>. Conversion of the biologically active EETs to the inactive metabolites, dihydroxyicosatetraenoic acids (DHETEs), by soluble epoxide hydrolase (sEH) has been reported in the SHR<sup>3</sup> (Fig. 1). Consistent with the latter findings, treatment of SHRs with inhibitors of sEH lowers blood pressure<sup>3,15</sup>. Therefore, modulation of AA hydroxylase activity (i.e., 20-HETE and EETs-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.

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<sup>16</sup> and further elaborated by Prochaska *et al.*<sup>17</sup>. Resveratrol (RES) is a natural phytochemical which is a phytoalexin commonly found in grapes, berries and peanuts. Several recent studies have shown that RES prevents or slows the progression of a wide variety of illnesses, including cancer, cardiovascular disease and ischemic injuries, as well as enhances stress resistance and extend the life spans of various organisms from yeast to vertebrates<sup>18</sup>. RES has been shown to have vasorelaxant effects through the stimulation of Ca<sup>2+</sup> activated K<sup>+</sup> channels and enhancing the nitric oxide signaling in the endothelium<sup>19,20</sup>. It has also been shown that the treatment of SHR with RES attenuated the hypertension through the modulation of nitric oxide synthase<sup>20</sup>. In addition, a recent meta-analysis on 247 patients by Liu *et al.*<sup>21</sup> on the effect of RES on blood pressure indicated that RES consumption significantly decreases systolic blood pressure at high doses ( $\geq 150$  mg day<sup>-1</sup>)<sup>21</sup>. While the data on the effects of RES on high blood pressure have been validated *in vitro* and *in vivo*, the mechanisms responsible for the effects remain to be fully elucidated. The current

study was undertaken to examine the anti-hypertensive effect of RES in a hypertensive rat model and to investigate its effect on AA-metabolizing enzymes in the kidney.

## MATERIALS AND METHODS

**Materials:** Resveratrol was purchased from TCI (Portland, OR). Lutrol F127 Pluronic® (F127) was kindly donated by BASF (Florham Park, NJ). Ethanol USP was purchased from VWR (Radnor, PA). 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 Life Span Biosciences, Inc. (Seattle, WA). Acetonitrile and methanol were of High Pressure Liquid Chromatography (HPLC) grade and obtained from Fisher Scientific (Pittsburg, PA). All other chemicals used were of analytical grade VWR (Radnor, PA).

**Animals:** Male 14-week-old Spontaneously Hypertensive Rats (SHR) were obtained from Harlan Laboratories (Madison, WI). 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 and Science University.

**Preparation of Resveratrol (RES) solutions:** RES stock solutions were prepared in pure ethanol at a concentration of 3% w/v. Lutrol F127 Pluronic®, a GRAS excipient recognized by the FDA was prepared at 5% w/v in DI water to keep the RES dissolved in the solution for the duration of the study. This concentration of F127 is commonly used in commercial products for human consumption at concentrations of 5% or below. Oral LD<sub>50</sub> in rats for F127 is >10 g kg<sup>-1</sup> <sup>22</sup> and at a 5% concentration, the daily dose of F127 ingested would be less than 10 g kg<sup>-1</sup>. RES solutions were prepared fresh (1 L at a time) to obtain final concentrations of 0.15 mg mL<sup>-1</sup> (low dose group) or 0.3 mg mL<sup>-1</sup> (high dose group). Considering the average rat weight and average daily water intake, these

concentrations provided approximately 20 and 40 mg kg<sup>-1</sup> RES, respectively. The final ethanol concentrations in the low dose and high dose group were 0.015 and 0.03%v/v, respectively. The amount of ethanol present in the solutions was well below the oral LD50 concentration of 10.6 g kg<sup>-1</sup> in rats<sup>23</sup>.

RES stability in the Pluronic® F127 formulations was assessed using UV absorbance on a Biotek Sunergy 2 microplate reader at 306 nm. Briefly, RES stock solutions in ethanol were prepared at 15 mg mL<sup>-1</sup> or 25 mg mL<sup>-1</sup> and diluted into water, 1, 2.5 or 5% Pluronic® F127 solutions for a final concentration of RES at 0.15 or 0.25 mg mL<sup>-1</sup>, respectively. The samples were stored at room temperature. Blank solutions of water, 1, 2.5 and 5% Pluronic F127 solutions were prepared to account for vehicle effects. Samples were assessed on day 1 (day of preparation) and day 3 following a 1:100 dilution in their respective vehicles. Absorbance was recorded at 306 nm. By day 4, samples showed precipitation of RES and no additional measurements were made. Based on the presented data the RES at low and high doses showed the highest stability in 5% Pluronic® F127 solution.

**Resveratrol(RES) treatment:** Following acclimation in the laboratory, 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 resveratrol in the drinking water at concentrations of 20 and 40 mg kg<sup>-1</sup>, respectively, for 7 weeks. Fresh RES solutions were made from stock twice a week. The selection of RES doses was based on preliminary studies and previous studies<sup>21,23</sup>.

**Mean Arterial Pressure (MAP) measurements:** Blood pressure was measured as previously described by Vermehren-Schmaedick *et al.*<sup>24</sup>. All blood pressure measurements were performed in conscious animals. For each SHR rat, blood pressure was measured once every week using a non-invasive 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). Previously, it have been indicated that blood pressure values obtained with the non-invasive tail-cuff method are not significantly different from the values obtained in the same animals by direct measurements in the femoral artery<sup>24</sup>. 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.

**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  $-80^{\circ}\text{C}$  until use.

**Preparation of microsomes and measurement of protein content:** Kidney microsomes were prepared as described previously<sup>25</sup>. Microsomal protein concentrations were determined in triplicate using bovine serum albumin as a calibration standard as described before<sup>26</sup>. Absorbance was measured at 750 nm on a Synergy2<sup>®</sup> micro-plate reader using Gen5 Software (BioTek, Winooski, VT).

**Microsomal Arachidonic Acid (AA) hydroxylation and analysis of 20-HETE formation:** Oxidation of Arachidonic Acid (AA) to its metabolite 20-HETE was determined in reaction mixtures (500  $\mu\text{L}$ ) containing 100 mM phosphate buffer (pH 7.4), 40  $\mu\text{M}$  AA, 2 mM  $\text{MgCl}_2$ , 1 mM NADPH and rat kidney microsomes equivalent to 0.4 mg protein. Reactions were initiated with NADPH and were terminated after 15 min at  $37^{\circ}\text{C}$  with 1.0 M HCl. *In vitro* experiments using renal cortical microsomes from untreated Sprague-Dawley rats were similarly utilized to study the effect of RES on 20-HETE formation *in vitro*. In these experiments, microsomes were exposed to different RES concentrations (1, 10 and 50  $\mu\text{M}$ ) in presence and absence of NADPH. 20-HETE formation rate in both *in vitro* and *in vivo* studies was measured as described previously<sup>27</sup> with little modifications. 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 C<sub>18</sub> column (4.6 $\times$ 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%  $\text{min}^{-1}$ ) was utilized over 30 min, followed by a sudden increase to 20%  $\text{min}^{-1}$  to 100% acetonitrile. Flow rate was maintained at 0.3  $\text{mL min}^{-1}$  and column temperature at  $40^{\circ}\text{C}$ .

**Immunoquantification of CYP4A in rat kidney microsomes:** Rat kidney microsomes (10 kg per lane,) were incubated at  $100^{\circ}\text{C}$  for 5 min in Laemmli sample buffer (BioRad, Hercules, CA) and electrophoresed for 1 h at constant voltage (150 V) through precast polyacrylamide gels (BioRad) with minor modifications<sup>28</sup>. Microsomal proteins were then

transferred electrophoretically (30 min at 20V) to PVDF membrane and incubated with CYP4A1/CYP4A2/CYP4A3 rabbit anti-rat polyclonal antibody, at a 1:1000 dilution (Lifespan Biosciences) overnight at  $4^{\circ}\text{C}$ . After washes with PBST, the secondary antibody (goat anti-rabbit conjugated to horseradish peroxidase, Thermo Scientific) was added at a 1:5000 dilution for 1 h at room temperature. The immunoreactive proteins were detected via enhanced chemiluminescence and x-ray film imaging and the resultant signals were analyzed by densitometry (Bio-Rad). The intensity of the band was normalized to GADPH signals, which was used as loading control.

**Epoxide Hydrolase activity (sEH):** Metabolism of Epoxy Fluor 7 (Cayman Chemical Co.) to a fluorescent metabolite was utilized to determine the sEH activity in kidney microsomes as described previously<sup>29</sup>. Briefly, reactions were carried out in mixtures (200  $\mu\text{L}$ ) containing 25 mM Bis Tris-HCl, 1 mg  $\text{mL}^{-1}$  BSA, Epoxy Fluor 7 and rat kidney microsomes (equivalent to 10  $\mu\text{g}$  protein). The resulting solution was incubated at  $37^{\circ}\text{C}$  in a black 96-well flat bottom plate. The fluorescence of the Epoxy Fluor 7 metabolite was determined using an excitation wavelength of 330 nm and emission wavelength of 465 nm on a Synergy2<sup>®</sup> microplate reader using Gen5 Software (BioTek).

**Data analysis:** Data is reported as Mean $\pm$ SD. Differences in 20-HETE formation rate, CYP activity, level of protein expression and differences in MAP among groups, were assessed by one-way Analysis of Variance (ANOVA) with Tukey's *post-hoc* test for pair-wise multiple comparisons. Correlation analysis was also performed to investigate whether a change in blood pressure corresponds to a change in CYP4A or sEH activities. Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Tukey *post hoc* test was used for pair-wise comparison. The  $p < 0.05$  was considered as statistically significant.

## RESULTS

Exposure of rats to different doses of resveratrol (RES) had no obvious adverse effect, such as behavioral changes or loss of appetite and the gain in body weight was not significantly different in treatment groups compared to control animals.

**Effect of resveratrol treatment on Mean Arterial Pressure (MAP):** To determine the effect of RES on

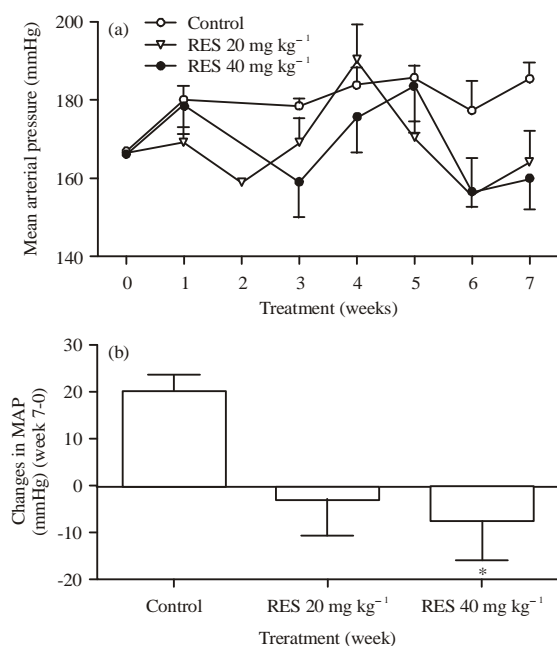


Fig. 2(a-b): Effect of RES on MAP in SHR rats. Male 14-week-old SHR rats ( $n = 5-7/\text{group}$ ) were exposed to RES in their drinking water at concentrations of 0, 20 and 40 mg kg<sup>-1</sup> for 7 weeks. MAP was measured non-invasively once every week. Change in MAP is expressed as Mean  $\pm$  SD of 5 (RES-treated) or 7 (control) animals. (a) Changes in MAP in SHR over 7 weeks and (b) Difference in MAP between the end and the beginning of the treatment, i.e., (MAP at week 7)-(MAP at week 0). \*Significant difference from control with  $p < 0.05$  (95% CI: 5.505-50.17)

blood pressure in SHR rats, the MAP of all groups was measured once weekly. Figure 2a shows the MAP in rats during baseline conditions and after exposure to low dose (20 mg kg<sup>-1</sup>) and high dose (40 mg kg<sup>-1</sup>) of RES. During the study, control rats showed a progressive rise in their MAP from  $166.3 \pm 1.9$  to  $185.4 \pm 10.8$  mm Hg after 7 weeks, an average increase of  $20.7 \pm 9.1$  mm Hg that represents 12.45% increase compared to baseline. In contrast, exposure of SHR rats to even the low dose of RES prevented the expected progressive rise in MAP with a net decrease of  $2.6 \pm 15.7$  mm Hg that represents 1.55% decrease compared to baseline. Moreover, administration of RES in the high dose resulted in a more substantial decrease in MAP from  $166.8 \pm 2.2$  at the beginning of the study to  $159.6 \pm 17.7$  at the end

of the 7 week treatment with a net decrease of  $7.2 \pm 19.2$  mm Hg or 4.22% decrease from the baseline. Although, both treatment groups showed MAP reduction at the end of the 7 week treatment compared to the control group, this reduction was only significant in the high-dose RES treatment group (One-way ANOVA  $p = 0.0017$ ; Tuckey's *post-hoc* test  $p < 0.05$  for control-high RES).

**Effect of RES treatment on arachidonic acid metabolism:** Previous studies have shown that CYP4A enzymes are entirely responsible for catalyzing the  $\omega$ -hydroxylation of (AA) to the potent vasoconstrictor metabolite, 20-HETE in the rat kidney<sup>12</sup>. To examine if the observed reduction in blood pressure in SHR rats by RES could be associated with inhibition of CYP4A-mediated formation of 20-HETE, this reaction was measured *in vitro* using renal cortical microsomes from untreated Sprague-Dawley rats. Microsomes were pre-incubated with or without RES (1, 10 and 50  $\mu\text{M}$ ) in the presence of NADPH before adding AA (100  $\mu\text{M}$ ) 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<sup>30</sup>. Under these chromatographic conditions, 20-HETE and AA were eluted with retention times of 36 and 38 min, respectively (Fig. 3b). Additionally as shown in Fig. 3a, 20-HETE formation was NADPH-dependent. This may indicate that inhibition of 20-HETE formation by RES is mechanism-based as NADPH was required in the incubation mixture for inactivation of CYP enzymes. The 20-HETE formation was inhibited by *in vitro* inactivation with RES in renal cortical microsomes in a concentration-dependent manner. At RES concentrations of 10 and 50  $\mu\text{M}$ , 20-HETE formation rate was reduced to 75 and 67% of control (Fig. 4a, *in vitro*).

The effect of the *in-vivo* administration of RES on AA metabolism was measured in rat renal microsomes after 7 weeks of exposure to RES in the drinking water at concentrations of 20 and 40 mg kg<sup>-1</sup>. Similar to the observed *in vitro* results, AA  $\omega$ -hydroxylation was inhibited by RES treatment, however this inhibition was only significant at the high-dose of RES compared to control rats, (Fig. 4b, *in vivo*).

**Effect of RES treatment on CYP4A protein levels:** To examine the effect of RES on the expression level of



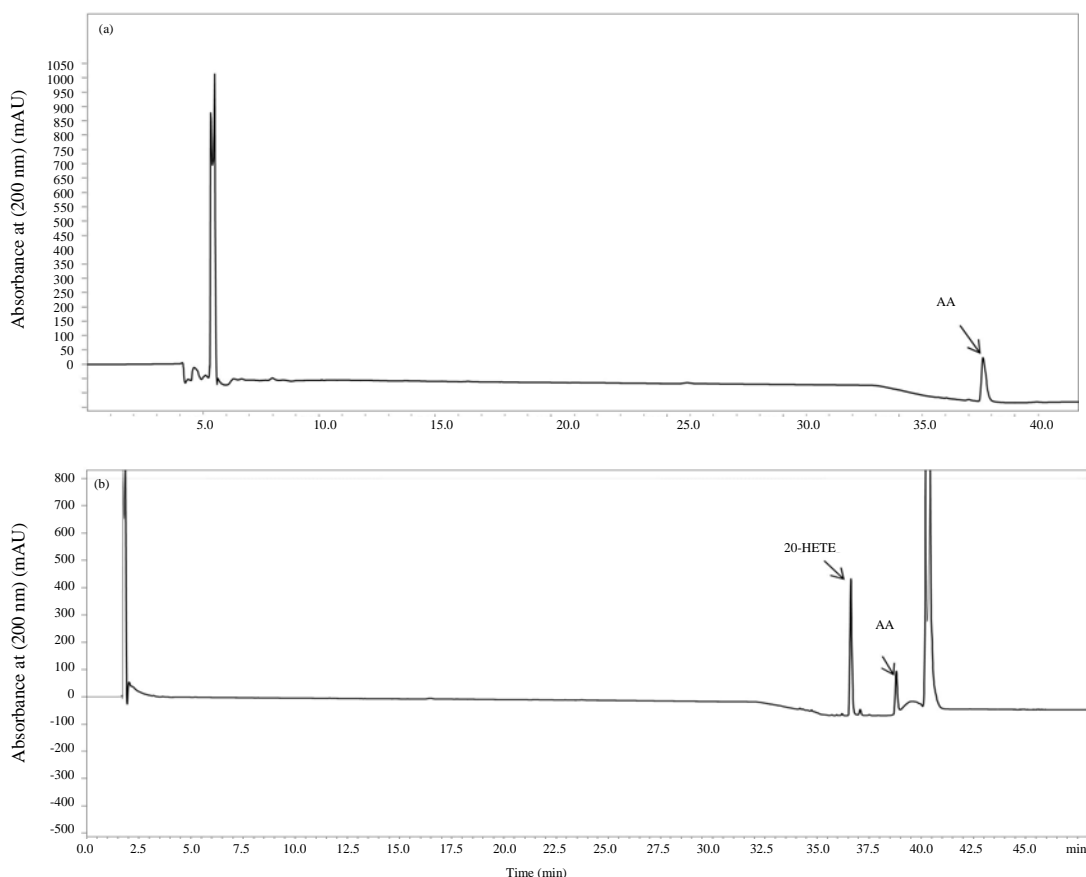


Fig. 3(a-b): HPLC analysis of AA metabolism by rat kidney microsomes representative chromatogram produced after incubation of AA with, (a) Untreated Sprague-Dawley rat kidney microsomes in the absence of NADPH and (b) Kidney microsomes from SHR treated with 20 mg kg<sup>-1</sup> RES in drinking water for 7 weeks

CYP4A protein, western blotting of renal microsomes was performed using a polyclonal antibody against rat CYP4A1/2/3. As shown in Fig. 5, RES-mediated inhibition of AA  $\omega$ -hydroxylation was associated with a loss of CYP4A immunoreactive protein. Similar to the observed dose-dependent inhibition of AA hydroxylase activity, RES also caused a significant inhibition of CYP4A protein expression compared to the control group but no significant difference was observed between the two RES treatment groups (Fig. 5).

**Effect of RES treatment on soluble epoxide hydrolase activity:** Several studies have provided compelling evidence that sEH plays a critical role in the metabolic conversion of the anti-hypertensive eicosanoids to inactive metabolites. To further study the effect of RES treatment on sEH activity, kidney microsomes from control rats or rats exposed to

different concentrations of RES were utilized as explained briefly in the “Materials and Methods” section<sup>29</sup>. Although the low dose RES did not have any effect on sEH, the high dose RES significantly inhibited the activity to 73% of the control value (Fig. 6).

As establishing a cause-effect relationship between the observed effect of RES on MAP, CYP4A and sEH activity was not possible with the presented study, the correlation between these variables was investigated. When RES-dependent changes in MAP were compared to changes in 20-HETE formation rate as a measure of CYP4A activity, a good correlation was observed (Pearson  $r$ ; -0.85 and  $p$ : 0.038). While 20 and 40 mg kg<sup>-1</sup> RES doses reduced MAP by approximately 2 and 9%, 20-HETE formation rate was reduced by 11 and 36%, respectively (Fig. 7). Similarly, changes in MAP reasonably correlate (Pearson  $r$ ; -0.75 and  $p$ : 0.046) with changes in sEH

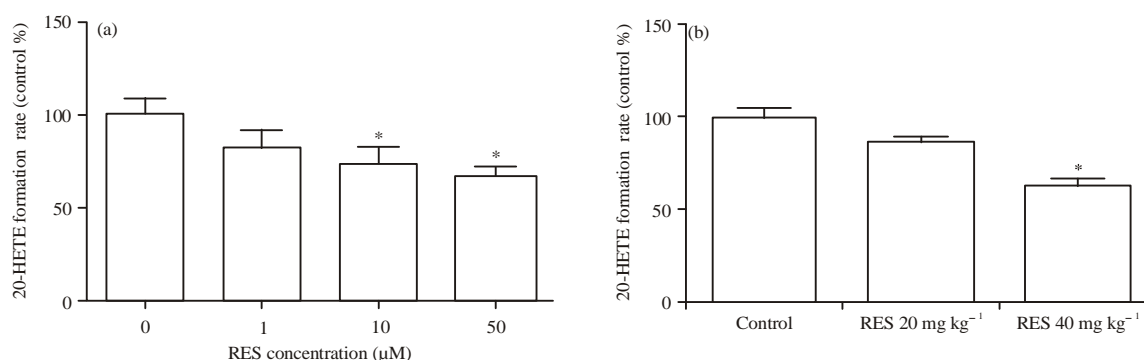


Fig. 4(a-b): Inhibition of AA  $\omega$ -hydroxylation activity by RES both *in vitro* and *in vivo* (a) Renal cortical microsomes from untreated Sprague Dawley rats were incubated with various concentrations of RES in the presence of NADPH. RES-treated microsomes were then used to measure 20-HETE formation. Values, average of 5 replicates, are expressed as % of control (RES-free microsomes) and (b) Male SHR rats ( $n = 5$ /group) were administered 20 or 40 mg kg<sup>-1</sup> RES in their drinking water for 7 weeks and kidneys were harvested at the end of the study. The NADPH-dependent formation of 20-HETE was measured in renal cortical microsomes. Values are expressed as % of control (rats that received RES-free water,  $n = 7$ ). All groups were compared using one-way ANOVA followed by multiple comparisons. \*Significant difference from control with  $p < 0.05$

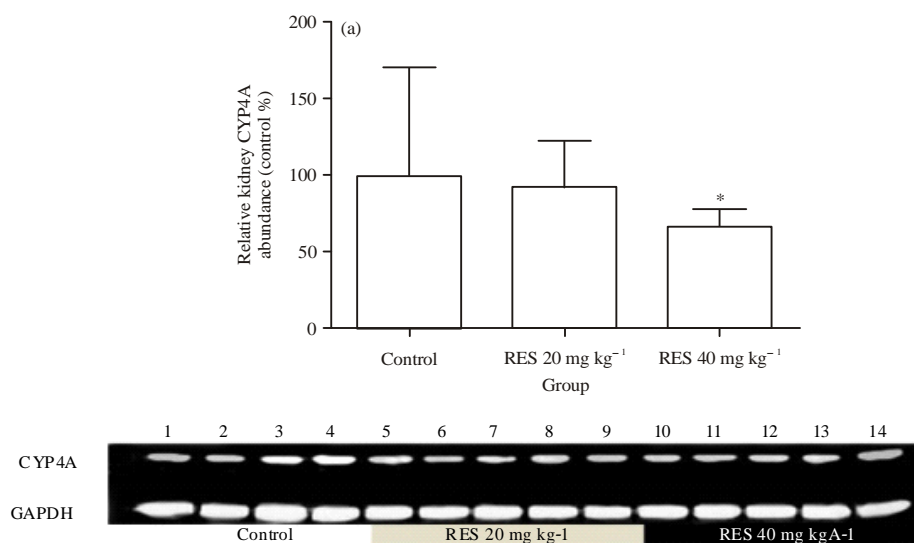


Fig. 5(a-b): Effect of RES on kidney expression of CYP4A. (a) Renal CYP4A protein levels in rats ( $n = 5-7$ /group) treated for 7 weeks with RES at 20 and 40 mg kg<sup>-1</sup>, expressed as the percentage of control levels in untreated rats. The immunoreactive proteins were detected via enhanced chemiluminescence and x-ray film imaging and the resultant signals were analyzed by densitometry. The intensity of the band was normalized to a loading control (GAPDH signals), then data was expressed as percent of control and (b) Original specimen analyzed by densitometry (Control; Lanes 1-4, Low RES dose: Lanes 5-9, High RES dose: Lanes 10-14). \*Significant difference from control with  $p < 0.05$

activity in a RES dose-dependent manner. Namely, compared to 2 and 9% reduction in MAP, sEH activity

was reduced by 5 and 25% following administration of 20 and 40 mg kg<sup>-1</sup> RES, respectively (Fig. 7).

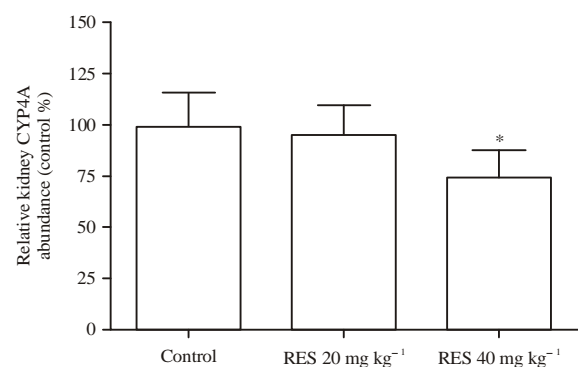


Fig. 6: Effect of RES on activity of kidney sEH Mean epoxide hydrolase (sEH) activity in rats ( $n = 5/\text{RES}$  and  $n = 7/\text{control}$  groups) treated for 7 weeks with RES at 20, 40 mg kg<sup>-1</sup>, expressed as the percentage of control fluorescence in rats that received RES-free water. Rat kidney microsomes (equivalent to 10  $\mu\text{g}$  protein) were incubated with Epoxy Fluor 7 and the fluorescence of the Epoxy Fluor 7 metabolite was determined. Data is presented as Mean  $\pm$  SD. \*Significant difference with  $p < 0.05$

## DISCUSSION

As previously demonstrated that sulforaphane, the main active isothiocyanate in cruciferous vegetable, reduced the progressive rise in blood pressure in Spontaneously Hypertensive Rats (SHR) through modulation of Arachidonic Acid (AA) metabolism<sup>30</sup>. This study was undertaken to investigate possible mechanisms that contribute to the beneficial effect of resveratrol (RES) on blood pressure in hypertensive rats. The results show for the first time that treatment of SHR with RES 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.

RES is a naturally-occurring compound that exhibits cytoprotective, antioxidant and anti-inflammatory vasoprotective properties<sup>31</sup>. Most studies examining the mechanism of action of RES proposed that preventing and/or inhibiting the development of cardiovascular diseases is related to its ability to reduce oxidative stress and associated inflammation and thus, ameliorate diseases that become more common with aging, such as hypertension<sup>23,31</sup>. For example, the consumption of 25 mg kg<sup>-1</sup> RES in drinking water for 14 days reduced

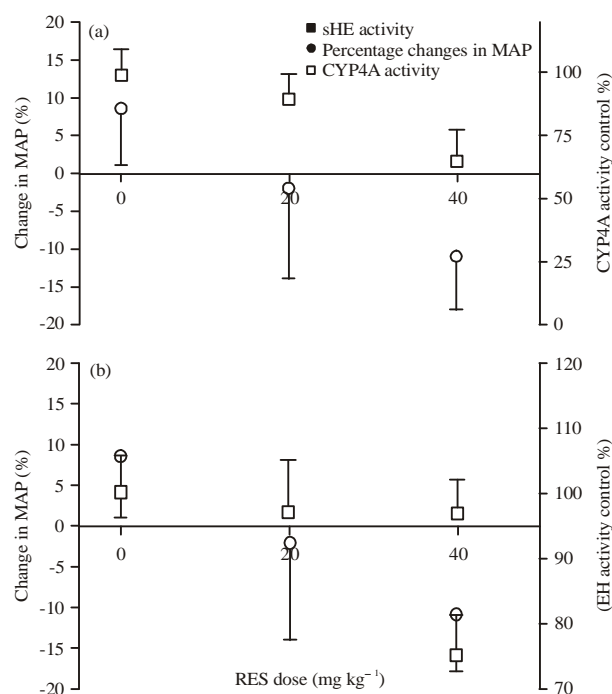


Fig. 7(a-b): Correlation of changes in MAP, CYP activity and sEH activity as a function of RES dose. Percent Changes in MAP (closed circles) in correlation to (a) CYP4A activity (open squares) and (b) sEH activity (closed squares) as a function of RES dose. MAP is presented as % change from baseline (week 0). Both CYP4A and sEH activities are presented as a % control activities. While treatment rats received RES in their drinking water in doses of 20 or 40 mg kg<sup>-1</sup> for 7 weeks, control rats did not receive RES

oxidative stress, improved endothelial functions and protected rats against development of pulmonary hypertension<sup>23</sup>. Furthermore, consumption of diets containing high dose RES by SHRs was found to improve vascular function, attenuate high BP and prevent cardiac hypertrophy.

Whether this anti-hypertensive and cardioprotective effect of RES is due to antioxidant effect is not yet established. Additionally, if the anti-hypertensive effects of RES are due to exclusively because of its antioxidant properties, then other antioxidants should also show similar anti-hypertensive properties. However, results of three randomized clinical trials testing the effects of the antioxidant vitamin E on hypertension have proven



disappointing<sup>32-34</sup>. Similarly, a non-significant reduction in blood pressure was reported in vitamin D-treated patients<sup>35</sup>. Accordingly, it is conceivable that RES reduces blood pressure by more than one mechanism.

This study is the first to investigate the effect of RES on modulation of AA-metabolizing enzymes in the kidney, as a potential mechanism for its anti-hypertensive 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. The 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 anti-hypertensive 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 down-regulation 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 RES administration was introduction RES to rats in their drinking water rather than oral gavage or subcutaneous injection. Stability studies using absorbance measurements indicate that RES is stable up to 0.25 mg mL<sup>-1</sup> in 5% Pluronic® F127 solutions at room temperature for 3 days. Notably, the observed attenuation of the expected rise in blood pressure by RES after a 7 week period was comparable to the results achieved with commonly used anti-hypertensive medication in the same rat model. For example, Telmisartan (5 mg for 8 weeks), Indapamide (1 mg kg<sup>-1</sup> for 8 weeks), Hydrochlorothiazide (20 mg kg<sup>-1</sup> for 8 weeks) and Metoprolol (100 mg kg<sup>-1</sup>) with Hydralazine (50 mg kg<sup>-1</sup> for 10 weeks) resulted in an average MAP reduction of 25, 17, 15 and 22 mm Hg; respectively<sup>36,37</sup>. 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<sup>3</sup>. 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<sup>12</sup>. The presented data are consistent with this hypothesis and with several findings that inhibitors of 20-HETE synthesis lower blood pressure by 10-15 mm Hg in

SHR<sup>13</sup>. In attempts to identify the cytochrome P450 (CYP) isoforms responsible for the renal metabolism of AA and formation of 20-HETE using selective inhibitors and anti-sense oligonucleotides, it has been identified that CYP4A1 exhibits the highest catalytic activity for the formation of 20-HETE in the rat kidney, followed by CYP4A2 and CYP4A3<sup>7,38</sup>. Using specific antibody against CYP4A1/2/3, the immunoblotting data indicate that RES lowers the expression of these isoforms in a manner similar to that involved in reducing MAP and 20-HETE formation.

It has also been established that EET metabolites of AA produced by the endothelium are potent vasodilators and natriuretic agents and are proposed as endothelium-derived hyperpolarizing factors<sup>39</sup>. Degradation of these metabolites by sEH led to more research interest towards inhibiting this enzyme as an avenue to increase EETs levels and prevent progression of hypertension<sup>40</sup>. In addition to elevated renal production of 20-HETE, SHRs also exhibit an increased expression of renal sEH compared to their normotensive WKY control rats<sup>41,42</sup>. Specific inhibitors to sEH enzyme lowered blood pressure in SHR, but not in WKY rats<sup>41</sup>. Such findings may indicate that sEH contributes to the initiation of mechanisms that eventually result in high blood pressure in SHR. The presented data indicate for the first time that both low and high RES doses inhibit the activity of sEH in the kidney of SHR and this inhibition was parallel to the observed reduction in blood pressure.

## CONCLUSION

The presented results have shown that even the lower RES dose prevented the progressive rise in MAP while the higher RES dose significantly reduced MAP in hypertensive rats. For the first time, it was demonstrated that the lowering of blood pressure was accompanied by modulation of AA metabolism through inhibiting both, the formation of 20-HETE and activity of sEH. Those results contribute the novel knowledge to the anti-hypertensive properties of RES.

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

## ACKNOWLEDGMENTS

We thank Bryan Bustamante for assistance in measuring blood pressure and Anik Amin, Margarita Can and Tamara Olenyik for microsomal preparation,

HPLC and immunoblotting assays. This study was supported in part by the Medical Research Foundation of Oregon (MRF), Collins Medical Trust and Faculty Development Grant from Pacific University, Oregon (F.E.).

## REFERENCES

- O'Brien, E., D.G. Beevers and H.J. Marshall, 1995. ABC of Hypertension. BMJ Publishing Group, London.
- Lasker, J.M., W.B. Chen, I. Wolf, B.P. Blowski, P.D. Wilson and P.K. Powell, 2000. Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney role of cyp4F2 and cyp4A11. *J. Biol. Chem.*, 275: 4118-4126.
- Sarkis, A. and R.J. Roman, 2004. Role of cytochrome P450 metabolites of arachidonic acid in hypertension. *Curr. Drug Metab.*, 5: 245-256.
- Roman, R.J., 2002. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol. Rev.*, 82: 131-185.
- Zhao, X. and J.D. Imig, 2003. Kidney CYP450 enzymes: Biological actions beyond drug metabolism. *Curr. Drug Metab.*, 4: 73-84.
- Makita, K., J.R. Falck and J.H. Capdevila, 1996. Cytochrome P450, the arachidonic acid cascade and hypertension: New vistas for an old enzyme system. *FASEB J.*, 10: 1456-1463.
- Maier, K.G. and R.J. Roman, 2001. Cytochrome P450 metabolites of arachidonic acid in the control of renal function. *Curr. Opin. Nephrol. Hypertension*, 10: 81-87.
- Zou, A.P., J.T. Fleming, J.R. Falck, E.R. Jacobs, D. Gebremedhin, D.R. Harder and R.J. Roman, 1996. 20-HETE is an endogenous inhibitor of the large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channel in renal arterioles. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.*, 270: R228-R237.
- Ma, Y.H., D. Gebremedhin, M.L. Schwartzman, J.R. Falck and J.E. Clark *et al.*, 1993. 20-Hydroxyeicosatetraenoic acid is an endogenous vasoconstrictor of canine renal arcuate arteries. *Circ. Res.*, 72: 126-136.
- Zou, A.P., J.D. Imig, M. Kaldunski, P.R.O. de Montellano, Z. Sui and R.J. Roman, 1994. Inhibition of renal vascular 20-HETE production impairs autoregulation of renal blood flow. *Am. J. Physiol. Renal Physiol.*, 266: F275-F282.
- Cooke, C.L.M. and S.T. Davidge, 2002. Peroxynitrite increases iNOS through NF-kappaB and decreases prostacyclin synthase in endothelial cells. *Am. J. Physiol.*, 282: C395-C402.
- Su, P., K.M. Kaushal and D.L. Kroetz, 1998. Inhibition of renal arachidonic acid  $\omega$ -hydroxylase activity with ABT reduces blood pressure in the SHR. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.*, 275: R426-R438.
- Campbell, W.B. and J.R. Falck, 2007. Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension*, 49: 590-596.
- Makita, K., K. Takahashi, A. Karara, H.R. Jacobson, J.R. Falck and J.H. Capdevila, 1994. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. *J. Clin. Invest.*, 94: 2414-2420.
- Pomposiello, S.I., J. Quilley, M.A. Carroll, J.R. Falck and J.C. McGiff, 2003. 5, 6-Epoxyeicosatrienoic acid mediates the enhanced renal vasodilation to arachidonic acid in the SHR. *Hypertension*, 42: 548-554.
- Wattenberg, L.W., 1975. Effects of dietary constituents on the metabolism of chemical carcinogens. *Cancer Res.*, 35: 3326-3331.
- Prochaska, H.J., M.J. de Long and P. Talalay, 1985. On the mechanisms of induction of cancer-protective enzymes: A unifying proposal. *Proc. Nat. Acad. Sci.*, 82: 8232-8236.
- Baur, J.A. and D.A. Sinclair, 2006. Therapeutic potential of resveratrol: The *in vivo* evidence. *Nat. Rev. Drug Discovery*, 5: 493-506.
- Li, H.F., S.A. Chen and S.N. Wu, 2000. Evidence for the stimulatory effect of resveratrol on  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  current in vascular endothelial cells. *Cardiovascular Res.*, 45: 1035-1045.
- Bhatt, S.R., M.F. Lokhandwala and A.A. Banday, 2011. Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in spontaneously hypertensive rats. *Eur. J. Pharmacol.*, 667: 258-264.
- Liu, Y., W. Ma, P. Zhang, S. He and D. Huang, 2015. Effect of resveratrol on blood pressure: A meta-analysis of randomized controlled trials. *Clin. Nutr.*, 34: 27-34.
- Wiberg, G.S., H.L. Trenholm and B.B. Coldwell, 1970. Increased ethanol toxicity in old rats: Changes in LD50, *in vivo* and *in vitro* metabolism and liver alcohol dehydrogenase activity. *Toxicol. Applied Pharmacol.*, 16: 718-727.
- Csiszar, A., N. Labinskyy, S. Olson, J.T. Pinto and S. Gupte *et al.*, 2009. Resveratrol prevents monocrotaline-induced pulmonary hypertension in rats. *Hypertension*, 54: 668-675.

24. Vermehren Schmaedick, A., V.K. Jenkins, H.Y. Hsieh, A.L. Brown, M.P. Page, V.L. Brooks and A. Balkowiec, 2013. Upregulation of brain derived neurotrophic factor expression in nodose ganglia and the lower brainstem of hypertensive rats. *J. Neurosci. Res.*, 91: 220-229.
25. Elbarbry, F.A., P.J. McNamara and J. Alcorn, 2007. Ontogeny of hepatic CYP1A2 and CYP2E1 expression in rat. *J. Biochem. Mol. Toxicol.*, 21: 41-50.
26. Fryer, H.J., G.E. Davis, M. Manthorpe and S. Varon, 1986. Lowry protein assay using an automatic microtiter plate spectrophotometer. *Anal. Biochem.*, 153: 262-266.
27. Powell, P.K., I. Wolf, R. Jin and J.M. Lasker, 1998. Metabolism of arachidonic acid to 20-hydroxy-5, 8, 11, 14-eicosatetraenoic acid by P450 enzymes in human liver: Involvement of CYP4F2 and CYP4A11. *J. Pharmacol. Exp. Ther.*, 285: 1327-1336.
28. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
29. Nelson, J.W., R.M. Subrahmanyam, S.A. Summers, X. Xiao and N.J. Alkayed, 2013. Soluble epoxide hydrolase dimerization is required for hydrolase activity. *J. Biol. Chem.*, 288: 7697-7703.
30. Elbarbry, F., A. Vermehren-Schmaedick and A. Balkowiec, 2014. Modulation of arachidonic acid metabolism in the rat kidney by sulforaphane: Implications for regulation of blood pressure. *Int. Scholarly Res. Notices*, Vol. 2014. 10.1155/2014/683508
31. Jiang, H., X. Shang, H. Wu, G. Huang and Y. Wang *et al.*, 2010. Combination treatment with resveratrol and sulforaphane induces apoptosis in human U251 glioma cells. *Neurochem. Res.*, 35: 152-161.
32. GISSI-Prevenzione Investigators, 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: Results of the GISSI-Prevenzione trial. *Lancet*, 354: 447-455.
33. Palumbo, G., F. Avanzini, C. Alli, M.C. Roncaglioni and E. Ronchi *et al.*, 2000. Effects of vitamin E on clinic and ambulatory blood pressure in treated hypertensive patients. *Am. J. Hypertension*, 13: 564-567.
34. Yusuf, S., G. Dagenais, J. Pogue, J. Bosch and P. Sleight, 2000. Vitamin E supplementation and cardiovascular events in high-risk patients. The heart outcomes prevention evaluation study investigators. *N. Engl. J. Med.*, 342: 154-160.
35. Witham, M.D., M.A. Nadir and A.D. Struthers, 2009. Effect of vitamin D on blood pressure: A systematic review and meta-analysis. *J. Hypertens*, 27: 1948-1954.
36. Chowdhury, G., M.W. Calcutt, L.D. Nagy and F.P. Guengerich, 2012. Oxidation of methyl and ethyl nitrosamines by cytochrome P450 2E1 and 2B1. *Biochemistry*, 51: 9995-10007.
37. Richards, T.R. and S.W. Tobe, 2014. Combining other antihypertensive drugs with  $\beta$ -blockers in hypertension: A focus on safety and tolerability. *Can. J. Cardiol.*, 30: S42-S46.
38. Holla, V.R., K. Makita, P.G. Zaphiropoulos and J.H. Capdevila, 1999. The kidney cytochrome P-450 2C23 arachidonic acid epoxygenase is upregulated during dietary salt loading. *J. Clin. Invest.*, 104: 751-760.
39. Chaudhry, A.P., K.P. Saigal, M. Intengan and P.A. Nickerson, 1979. Malakoplakia of the large intestine found incidentally at necropsy: Light and electron microscopic features. *Dis. Colon Rectum*, 22: 73-81.
40. Imig, J.D., 2005. Epoxide hydrolase and epoxygenase metabolites as therapeutic targets for renal diseases. *Am. J. Physiol. Renal Physiol.*, 289: F496-F503.
41. Sacerdoti, D., B. Escalante, N.G. Abraham, J.C. McGiff, R.D. Levere and M.L. Schwartzman, 1989. Treatment with tin prevents the development of hypertension in spontaneously hypertensive rats. *Science*, 243: 388-390.
42. Yu, Z., L.M. Huse, P. Adler, L. Graham, J. Ma, D.C. Zeldin and D.L. Kroetz, 2000. Increased CYP2J expression and epoxyeicosatrienoic acid formation in spontaneously hypertensive rat kidney. *Mol. Pharmacol.*, 57: 1011-1020.