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INDUCTION OF RENAL CYTOCHROME P450 ARACHIDONIC ACID EPOXYGENASE ACTIVITY BY DIETARY γ-LINOLENIC ACID

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Running Title: Dietary Induction of CYP Epoxygenases

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List of Non-standard Abbreviations:

PUFAs, polyunsaturated fatty acids; GLA, γ-linolenic acid; BOR, borage oil; SES, sesame oil; EETs, epoxyeicosatrienoic acids; 20-HETE, 20-hydroxyeicosatetraenoic acid; DHETs, dihydroxyeicosatrienoic acids; 10(11)-EpHep,10(11)-epoxyheptadecanoic acid; 10,11-DiHN,

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10,11-dihydroxynonadecanoic acid; BHT, butylated hydroxytoluene; SHR spontaneously hypertensive rat; WKY, Wistar Kyoto rat; CYP, cytochrome P-450; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography tandem mass spectrometry; TGF, tubuloglomerular feedback; SMC, smooth muscle cell.

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ABSTRACT

Dietary γ-linolenic acid (GLA), an ω-6 polyunsaturated fatty acid found in borage oil (BOR) lowers systolic blood pressure in spontaneously hypertensive rats (SHRs). GLA is converted into arachidonic acid (AA) by elongation and desaturation steps. Epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE) are cytochrome P450 (CYP) derived AA eicosanoids with important roles in regulating blood pressure. This study tested the hypothesis that the blood pressure lowering effect of a GLA-enriched diet involves alteration of CYP-catalyzed AA metabolism. Microsomes and RNA were isolated from the renal cortex of male SHRs fed a basal fat-free diet for 5 wks to which 11% by weight of sesame oil (SES) or BOR was added. There was a 2.6- to 3.5-fold increase in CYP epoxygenase activity in renal microsomes isolated from the BOR fed SHRs compared to the SES fed rats. Epoxygenase activity accounted for 58% of the total AA metabolism in the BOR treated kidney microsomes compared to 33% in the SES treated rats. More importantly, renal 14,15- and 8,9-EET levels increased 1.6- to 2.5-fold following dietary BOR treatment. The increase in EET formation is consistent with increases in CYP2C23, CYP2C11 and CYP2J protein levels. There were no differences in the level of renal CYP epoxygenase mRNA between the SES and BOR treated rats. Enhanced synthesis of the vasodilatory EETs and decreased formation of the vasoconstrictive 20-HETE suggests that changes in CYP-mediated AA metabolism may contribute, at least in part, to the blood pressure lowering effect of a BOR-enriched diet.

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INTRODUCTION

Hypertension is an important risk factor for stroke and coronary heart disease. In the United States, at least 31% of adults have hypertension and the total prevalence is increasing at dramatic rates (Fields et al., 2004). Hypertension is a multifactorial disease associated with cellular alterations in receptor function, signal transduction, ion transport, calcium mobilization and membrane potential. As integral components of cell membranes and important signaling molecules, lipids play an important role in the regulation of numerous cellular processes. Several studies have demonstrated abnormalities in lipid metabolism associated with hypertension. In the spontaneously hypertensive rat (SHR), the composition and metabolism of polyunsaturated fatty acids (PUFAs) in serum and tissues are altered prior to the onset of hypertension, indicating that an imbalance of individual PUFAs might contribute to the increase in blood pressure (Singer et al., 1983). On the other hand, dietary PUFA supplements can ameliorate high blood pressure in both animals and humans (Singer et al., 1990; Hornych et al., 2002).

Of particular interest is γ –linolenic acid (GLA), an ω-6 polyunsaturated fatty acid (18:3n-6). Borage oil (BOR) is a rich source of GLA (24%, volume/volume). In a rat 5/6 renal ablation model, a BOR diet resulted in a marked decrease in blood pressure, significantly less glomerulosclerosis, and an increase in renal arachidonic acid levels compared to corn oil-treated rats (Ingram et al., 1996). In both pre-hypertensive and hypertensive SHRs, dietary BOR significantly reduces systolic blood pressure (Engler et al., 1992; Engler et al., 1998). Further studies have shown that the anti-hypertensive effect of BOR is accompanied by an increase in endogenous GLA levels in plasma, liver and the vasculature (Engler and Engler, 1998). Similar cardiovascular protective effects have been reported following GLA treatment in salt-loaded

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borderline hypertensive rats (Mills et al., 1989). In humans, supplementation with GLA for 3 months is associated with a beneficial reduction of cardiovascular risk factors and stable renal function (Hornych et al., 2002). Despite numerous studies on the physiological effects of dietary GLA supplementation, little is known about the effect of this diet on the synthesis of biologically active metabolites derived from GLA.

GLA is either taken up directly from the diet or converted from the essential fatty acid linoleic acid by a Δ6-desaturase catalyzed desaturation step and can be converted through chain elongation and desaturation steps into arachidonic acid (Mathews et al., 2000). Metabolites of arachidonic acid are highly potent regulatory molecules with important roles in inflammation, blood pressure control and platelet aggregation.

The major cytochrome P450 (CYP)-derived eicosanoids are regio- and stereoisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE). EETs are further converted by soluble epoxide hydrolase (sEH) into corresponding dihydroxyeicosatrienoic acids (DHETs) (Kroetz and Zeldin, 2002). CYP eicosanoids function as autocrine and paracrine factors and mediate local cellular responses, including regulation of renal vascular tone, tubuloglomerular feedback (TGF), and sodium reabsorption (Kroetz and Zeldin, 2002). The formation of 20-HETE is mediated mainly by enzymes of the CYP4A and CYP4F family and 20-HETE has vasoconstrictive, natriuretic and diuretic properties (Kroetz and Zeldin, 2002). Epoxidation of arachidonic acid is catalyzed largely by members of the CYP2C (Karara et al., 1993) and CYP2J (Wu et al., 1997) families. Once formed, EETs can be either incorporated into membrane phospholipid pools or efficiently hydrolyzed to less active DHETs and secreted into the extracellular space (Zeldin et al., 1993). EETs have vasodilatory and natriuretic properties and are putative endothelium-derived

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hyperpolarizing factors (Hecker et al., 1994; Campbell et al., 1996). EETs also have anti-inflammatory (Node et al., 1999) and fibrinolytic properties (Node et al., 2001). The critical role of EETs and 20-HETE in the control of renal arteriole diameter and sodium reabsorption suggests an important contribution to integrated renal function and regulation of blood pressure.

In light of the vasoactive and renal regulatory roles of CYP eicosanoids, it is reasonable to postulate that dietary GLA could affect CYP eicosanoid levels and alter blood pressure. This hypothesis is supported by studies showing altered CYP-catalyzed arachidonic acid metabolism in multiple models of hypertension (Sarkis and Roman, 2004). While the beneficial effect of dietary PUFAs on cardiovascular disease is well recognized, there is a paucity of data on the molecular and/or genetic basis for the effect of GLA on blood pressure. In this study, we determined the effect of a GLA-enriched diet on renal arachidonic acid metabolism and renal CYP and sEH expression. The novel finding of induction of vasoprotective EET formation by GLA treatment provides a mechanism for the antihypertensive effects of a GLA-rich diet.

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METHODS

Materials. Radiolabeled nucleotides were purchased from New England Nuclear (Boston, MA) and radiolabeled arachidonic acid from Amersham Life Science (Arlington Heights, IL). Restriction enzymes were obtained from New England Biolabs (Beverly, MA), modifying enzymes from Gibco/BRL (Gaithersburg, MD), and RNase from Ambion (Austin, TX). All molecular biology grade chemicals, HPLC solvents, and ScintiVerse LC were from Fisher Scientific (Pittsburgh, PA). Nitrocellulose membranes were from Micron Separations (Westborough, MA). 15(S)-HETE-d8 and all regioisomeric EETs and DHETs were purchased from Cayman Chemicals (Ann Arbor, MI). All other reagents were of the highest grade available and were purchased from Fisher Scientific or Sigma Chemical Company.

Animals and dietary preparation. Details of the GLA diet and effects on blood pressure have been described previously (Engler and Engler, 1998). Male SHRs (14-15 weeks old) were obtained from Harlan (Indianapolis, IN) and housed two to three per cage at constant temperature (26°C) with 12 hr light/dark cycles. The rats were randomly assigned to two groups and fed a diet for 5 weeks of a fat-free basal mix (Teklad, Madison,WI) with the addition of 11% by weight of either sesame oil (SES) (Amend Drug & Chemical Co., Irvington, NJ) or borage oil (BOR) (Traco Labs, Seymour, IL). The oils were used to provide similar proportions of total 18-carbon monounsaturated and polyunsaturated fatty acids in the diet yet control for GLA, 18:3n6. The content of GLA in BOR was 24.4%. The control SES-based diet was rich in oleic acid (18:1n9; 35%) and devoid of GLA. At the end of the 5 week dietary treatment, anesthesia was administered with a mixture of oxygen (70%), nitrous oxide (30%) and halothane (5%). The abdominal cavities were opened, and the kidneys were perfused with ice-cold saline. Perfused kidneys were rapidly removed, and the cortex was dissected out and immersed in liquid nitrogen.

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All tissue was stored at -80˚C until preparation of RNA or microsomes. Animal use was approved by the University of California San Francisco Committee on Animal Research and followed the National Institutes of Health guidelines for the care and use of laboratory animals.

Renal arachidonic acid metabolism. Microsomes were prepared from frozen cortex using differential centrifugation and stored at -80˚C. Renal cortical arachidonic acid metabolism was measured in incubations containing [1-^{14}C]-arachidonic acid (85 µM, 0.2 µCi) using reverse phase HPLC with radiometric detection as described previously (Kroetz et al., 1997; Su et al., 1998; Yu et al., 2000a; Yu et al., 2000b; Xu et al., 2004).

Extraction surrogates and internal standard. 10(11)-epoxyheptadecanoic acid [10(11)-EpHep] and 10,11-dihydroxynonadecanoic acid (10,11-DiHN), kindly provided by Dr. John W. Newman (University of California, Davis), serve as EET and DHET extraction surrogates for liquid chromatography tandem mass spectrometry (LC/MS/MS) quantification of renal eicosanoids. The validation and synthesis of these standards have been previously described (Newman et al., 2002). Surrogate recoveries were calculated relative to the internal standard, 15(S)-HETE-d8. Calibration solutions comprised of eicosanoid standards (5-1500 µM) were prepared in acetonitrile, sealed and stored under nitrogen at –20°C.

Lipid extraction and hydrolysis. Frozen tissues were thawed on ice and approximately 100 mg of kidney cortex was used for lipid extraction. Butylated hydroxytoluene (BHT; 0.05g/L) was added during the extraction step to prevent peroxyl radical propagated transformations of PUFAs. An equal volume of methanol, two volumes of chloroform and extraction surrogates were added to the tissue, followed by homogenization and vortex mixing. The mixture was extracted twice with chloroform and the combined organic phase was washed once with distilled water. After evaporation of organic solvent under nitrogen, the dry residue

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was dissolved in 1N sodium hydroxide (1 ml) and incubated at room temperature for at least 3 hours to hydrolyze the fatty acids from the phospholipid backbone. Lipids were extracted twice with ethyl acetate, evaporated to dryness under a stream of nitrogen and stored at -80˚C until LC/MS/MS analysis. Immediately before analysis, the lipid residue was dissolved in 90 µl acetonitrile and supplemented with 10 µl of 15(S)-HETE-d8 (10 ng) as an internal control. A 10 µl sample aliquot was injected for analysis.

HPLC and mass spectrometry instrumentation. Analysis of lipids by HPLC was performed using a 2.0 x 150 mm, 5 µm Luna C18(2) column (Phenomenex) held at room temperature. Lipids were eluted from the reverse-phase HPLC column with acetonitrile/methanol/water/acetic acid, (55/10/35/0.01) at 0.8 ml/min. The effluent was introduced onto a Quattro Ultima tandem-quadrupole mass spectrometer (Micromass, Manchester, UK) and subjected to negative mode electrospray ionization (ESI) with a capillary voltage at –3.0 kV. Collision voltage ranged from –13 to –26 eV and cone voltage ranged from –50 to –60 V for EETs, DHETs and 20-HETE, optimized for sensitivity and inter-experiment consistency. Multi-reaction monitoring was used to simultaneously detect the four regioisomeric EETs and corresponding DHETs, 20-HETE and internal controls. Characteristic mass transition monitoring was adopted from the assay developed by the Hammock laboratory (Newman et al., 2002) with the exception of 15(S)-HETE-d8, for which the $[M\text{-}H]^{-} \rightarrow [Fragment]^{-}$ transition was monitored from m/z 327.2 to 182.3.

Ribonuclease protection assays. Total RNA was isolated from frozen renal cortex tissue by acid-phenol extraction. Construction of specific CYP4A, CYP2C23, CYP2E1, CYP2J3, CYP2J4, mEH and sEH riboprobes and details of the ribonuclease protection assays were described previously (Kroetz et al., 1997; Yu et al., 2000a; Yu et al., 2000b). The CYP4A3

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probe is used to detect both the CYP4A3 and CYP4A2 mRNA transcripts. A rat GAPDH ribonuclease protection probe was included as a control in all hybridizations. RNase protection assays were carried out as described earlier with the exception that [α-^{32}P]-UTP was used in place of [α-^{32}P]-CTP for the CYP2C23 and CYP2E1 probes. Gels were dried and protected fragments were visualized with a PhosphorImager and analyzed using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Western immunoblotting. Renal cortical microsomes (10 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose in 25 mM Tris/192 mM glycine/20% methanol using a semidry transfer system (BioRad, Hercules, CA). Primary antibodies used in these studies were a rabbit anti-rat CYP2C23 antisera that was a gift from Dr. Jorge H. Capdevila (Vanderbilt University, Nashville, TN) and a rabbit anti-human CYP2J2 IgG kindly provided by Dr. Darryl C. Zeldin (National Institute of Environmental Health Sciences). Goat anti-rat CYP4A1 and anti-rat CYP2C11 antisera were obtained from Gentest (Woburn, MA). A rabbit anti-mouse sEH antisera was kindly provided by Dr. Bruce Hammock (University of California, Davis) and a goat actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were incubated with a 1:5,000 (CYP2C23), 1:2,000 (CYP2C11), 1:3,000 (CYP2J2), 1:1,000 (CYP4A1) or 1:2000 (sEH) fold dilution of the primary antibody followed by a 1:10,000-fold dilution of Alexa Fluor 680 Donkey anti-Goat IgG or IRDye 800 Conjugated Affinity Purified anti-Rabbit IgG. Immunoreactive proteins were visualized and quantitated using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). CYP and sEH immunoreactive protein levels were expressed relative to the level of actin quantitated on the same blots.

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Statistics. Arachidonic acid metabolism and endogenous fatty acid quantification were performed in duplicate samples from individual animals and the results are expressed as mean ± SEM of five animals per treatment group. Statistical significance of differences between treatment groups was evaluated by an analysis of variance with multiple comparison testing with a modified *t* test. A value of $p < 0.05$ was considered to be statistically significant.

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RESULTS

Renal arachidonic acid metabolism in vitro. Arachidonic acid metabolism was measured in renal cortical microsomes from SES- and BOR-fed rats. Epoxygenase activities are expressed as the sum of EET and DHET formation, since epoxygenase activity is a prerequisite for DHET formation. All reactions were performed under conditions that were linear with respect to protein and time, and the formation of all metabolites was NADPH dependent (data not shown). The formation of 19-HETE increased 1.5-fold, and epoxide formation increased 3.6-fold in the kidneys of BOR-fed rats compared to the SES group, while 20-HETE formation was unchanged (Figure 1A). The increase in EET formation in BOR-treated rats was similar for the 8,9-, 11,12- and 14,15-EET regioisomers (Figure 1B). In both SES- and BOR-treated rats, renal epoxygenase activity is highest for the 11,12-EET regioisomer. Interestingly, the contribution of 19- and 20-HETE to total renal CYP eicosanoid formation decreased in the BOR-treated rats (15 vs. 11% for 19-HETE, and 51 vs. 31% for 20-HETE; Table 1). In contrast, EET formation was increased from 34% in SES-fed rats to 58% in BOR-fed rats, indicating that arachidonic acid is shunted toward the CYP epoxygenase pathway during BOR diets. Total renal arachidonic acid metabolism increased 2-fold in BOR-fed rats relative to SES-fed rats.

Renal EET levels. Whereas *in vitro* studies are important to characterize metabolic pathways of arachidonic acid metabolism, they cannot address many critical steps related to arachidonic acid metabolism in vivo. Endogenous CYP eicosanoid levels in the kidney cortex were quantified by LC-tandem mass spectrometry to determine the effect of SES and BOR diets on cellular levels of EETs within the kidney. Because the 5,6-EET metabolite is extremely labile and suffers extensive decomposition during sample preparation, its levels were not quantified in this study (Capdevila et al., 1981). The amount of each regioisomeric DHET is only about 10%

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of their corresponding EETs. The cortical 11,12- and 8,9-DHET levels are 1.7 fold higher in the BOR-fed rats compared to the control diet (Figure 2A). Endogenous 14,15-EET levels increased 1.6-fold and 8,9-EET content increased 2.5-fold in the renal cortex of BOR-fed relative to SES-fed SHRs (Figure 2B; $p < 0.01$). Although, the renal 11,12-EET levels remain unchanged between the two groups, increased formation of 11,12-EET is evident from the higher renal 11,12-DHET content in BOR-fed rats. The extent of increase in EET cellular content is consistent with the increase in CYP epoxygenase activity.

Renal CYP arachidonic acid epoxygenase and EH protein expression. The expression of CYP epoxygenases was characterized to explore the mechanistic basis for the increased epoxygenase activity in the BOR-fed SHR kidney. CYP2C and CYP2J protein levels were examined in cortical microsomes from SES- and BOR-fed SHRs by Western blotting and the expression level of these CYPs was quantitated and expressed relative to actin. Renal cortical CYP2C23 and CYP2C11 immunoreactive protein levels were 20-25% higher in BOR-fed SHRs compared to controls (Figure 3). An antisera directed against the human CYP2J2 protein is known to cross-react with rat CYP2J proteins (Yu et al., 2000a) and detected a 40% increase in CYP2J immunoreactive protein in kidneys from the BOR-fed SHRs compared with the SES-fed rats. Increased levels of CYP2C23, CYP2C11 and CYP2J immunoreactive protein are consistent with the increased arachidonic acid epoxygenase activity and endogenous EET levels in the kidney cortex of BOR-fed SHRs. Peptide-based antisera against CYP2J4 and CYP2J9 detect rat CYP2J4 and CYP2J3 proteins respectively, the two rat CYP2J isoforms currently identified. However, neither of these antisera detected differences in expression of CYP2J immunoreactive proteins between SES- and BOR-fed SHRs (data not shown). Thus, the identity of the CYP2J immunoreactive protein induced by a BOR diet remains unknown. The

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BOR diet had no effect on the levels of CYP4A immunoreactive proteins (Figure 3), consistent with the lack of effect of the BOR diet on CYP ω-hydroxylase activity. Expression of renal sEH was also increased following the GLA-enriched diet, which would contribute to the elevated DHET levels in BOR-fed SHR kidneys.

Renal CYP arachidonic acid epoxygenase and EH mRNA levels. RNase protection assays were used to quantitate the corresponding levels of CYP2C23, CYP2J3, CYP2J4, CYP2E1, CYP4A, mEH and sEH mRNA in the renal cortex of SES- and BOR-fed SHRs. The specificity of each assay was confirmed in preliminary studies with sense RNA transcribed from the full-length cDNAs (data not shown). Despite the increased CYP2C and CYP2J protein levels in BOR-fed SHRs, there were no measurable differences in the mRNA levels of any CYP epoxygenase genes after treatment with BOR (Figure 4). Furthermore, quantitation of CYP2C11 mRNA by Taqman RT-PCR demonstrated similar levels in the kidneys of SES- and BOR-fed SHRs (data not shown). These data suggest that the increased level of the CYP2C23, CYP2C11 and CYP2J2 immunoreactive proteins in the kidneys of BOR-fed SHRs is not a consequence of increased transcription and/or stabilization of the corresponding CYP epoxygenase mRNAs. Consistent with no change in CYP4A protein levels, the renal mRNA levels of CYP4A1, CYP4A2, CYP4A3 and CYP4A8 are comparable between rats receiving different dietary treatments (data not shown).

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DISCUSSION

We have previously reported that blood pressure is decreased in BOR-fed SHRs (Engler et al., 1998). The current analysis demonstrates that renal CYP epoxygenase-mediated arachidonic acid metabolism is elevated following a diet rich in GLA, suggesting that an increase in vasoprotective EETs might contribute to the blood pressure lowering effects of this diet. The rat kidney is deficient in Δ6-desaturase (Huang et al., 1994), the enzyme necessary to convert linoleic acid to GLA, but it can catalyze the conversion of GLA to arachidonic acid. It is possible that the anti-hypertensive effect of GLA resides in its ability to bypass the defective Δ6-desaturase pathway in the kidney. Thus, dietary supplementation with GLA increases arachidonic acid levels in the kidney, thereby providing increased substrate for CYP-catalyzed metabolism. Indeed, we observed a significant increase in endogenous EET levels in the kidney and in renal cortical CYP epoxygenase activity. This is consistent with the increase in arachidonic acid and prostaglandin levels in a rat renal failure model fed a BOR diet (Ingram et al., 1996).

Extensive investigation in recent years has established a role for cytochrome P450 eicosanoids in the regulation of blood pressure (Kroetz and Zeldin, 2002). CYP eicosanoids are synthesized throughout the body but act in a paracrine and autocrine fashion to regulate cellular function. The kidney produces significant levels of CYP eicosanoids with effects on renal tubular transport function and vascular reactivity and altered levels of renal EETs and HETEs are associated with changes in blood pressure (Su et al., 1998; Yu et al., 2000b; Xu et al., 2002). For these reasons, the current study focused on renal mechanisms that might contribute to the blood pressure lowering effects of a GLA-enriched diet. Essential fatty acid deficiency exacerbates hypertension in the SHR (Church JP, 1977) and there is a lower percentage of arachidonic acid

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in the SHR kidney relative to the Wistar Kyoto (WKY) (Delachambre et al., 1998). Phospholipid acyl-transferase activity and Δ6- and Δ5-desaturase activity are also decreased in the SHR relative to the WKY (Narce and Poisson, 1995). This low arachidonic acid content in the SHR membrane phospholipid pool might limit the synthesis of anti-hypertensive EETs in the kidneys and vasculature. A BOR diet is associated with increased levels of renal arachidonic acid (Ingram et al., 1996). However, numerous eicosanoids can be synthesized from arachidonic acid, often with opposing physiological effects. It is therefore difficult to predict the effect of increasing arachidonic acid pools on eicosanoid levels. Our data indicate an increase in all regioisomeric EETs in BOR fed SHRs, consistent with the broad inducibility of multiple CYP epoxygenases.

It is of interest to note that the renal microsomal epoxygenases catalyzed the highly asymmetric epoxidation of arachidonic acid to generate EETs, with a preference for 11,12-EET, and then 8,9- and 14,15-EET. This is consistent with data in the literature (Holla et al., 1999; Yu et al., 2000a) and demonstrates the characteristic renal epoxygenase regioselectivity in rats. In contrast, the regiochemical composition of renal endogenous EETs was different from the regioselectivity observed *in vitro*. The major endogenous regioisomeric EET in kidneys is 14,15-EET, followed by lower amounts of 8,9-EET and 11,12-EET in the SES-fed rats. The fact that the endogenous 14,15-EET content in rat kidney is the highest among all regioisomers is consistent with similar measurements in SHR urine (Yu et al., 2000a), and human liver (Zeldin et al., 1996), heart (Wu et al., 1996) and kidney (Karara et al., 1990). Furthermore, in BOR-fed SHRs, renal endogenous 8,9-EET levels increased more than the levels of other regioisomers and 8,9-EET becomes the major endogenous EET. This is in contrast with the parallel increase in the formation of all regioisomeric EETs in renal microsomes. The apparent discrepancy between *in*

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vitro activity and *in vivo* quantification might be due to the heterogeneous distribution of CYP epoxygenases in the kidney and /or unequal availability of free arachidonic acid throughout the kidney as compared with kidney microsomal incubations, where enzyme kinetic properties are the only determinant of metabolite formation from exogenously added arachidonic acid. In addition, CYP arachidonic acid metabolism is shunted from a predominant production of the prohypertensive 20-HETE to the antihypertensive EETs, consistent with the decrease in blood pressure associated with GLA treatment.

Several lines of evidence suggest a role for CYP epoxygenase activity in the regulation of blood pressure. CYP epoxygenase activity is increased in animal models of hypertension, including the SHR (Yu et al., 2000a) and a high salt diet (Makita et al., 1994). The elevation in EET biosynthesis could be a consequence of the elevated blood pressure and might represent a compensatory response of the animal to deleterious increases in blood pressure. Moreover, inhibition of epoxygenases by clotrimazole leads to salt-dependent hypertension (Holla et al., 1999) and an inability to increase CYP epoxygenase activity with excess dietary salt intake in Dahl salt-sensitive rats might contribute to the hypertensive phenotype of these animals (Makita et al., 1994). Down regulation of CYP2C23 and CYP4A expression is associated with obesity-induced hypertension (Wang et al., 2003). The induction of CYP2C and CYP2J expression by a BOR diet in parallel with a decrease in blood pressure in BOR-fed SHRs supports a protective role for CYP epoxygenase-mediated arachidonic acid metabolism in cardiovascular disease. Future studies utilizing CYP epoxygenase inhibitors and antisense oligonucleotides could provide additional evidence for an antihypertensive effect of a BOR-mediated induction of renal EET formation. It is also of interest to examine whether the inductive effects of the BOR diet on renal epoxygenase activity are consistent throughout the development of hypertension in the

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SHR and whether early feeding of this diet can prevent the hypertensive phenotype. It is exciting to speculate that pharmacologic modulation of CYP epoxygenase activity might be a novel therapeutic target for blood pressure control.

In addition to influencing blood pressure, dietary GLA can suppress smooth muscle cell (SMC) proliferation in vivo and retard the development of diet-induced atherosclerosis in apoE knockout mice (Fan et al., 2001). As recently demonstrated, EETs possess anti-inflammatory properties and decrease leukocyte adhesion to the vascular wall (Node et al., 1999), inhibit SMC migration (Sun et al., 2002), and reduce the formation of thrombi (Node et al., 2001). The anti-inflammatory and fibrinolytic activity of EETs may lower the risk of cardiovascular disease and partially explain the beneficial effect of dietary PUFAs in preventing atherosclerosis.

In this study, we detected elevations in the protein levels of CYP2C23, CYP2C11 and CYP2J2 immunoreactive proteins. Changes in CYP epoxygenase protein levels have also been documented in other studies, including an increase in response to a high salt diet (Holla et al., 1999) and a decrease in response to a high fat diet (Wang et al., 2003). The mechanism underlying these protein expression changes has not been addressed. Many CYP enzymes are transcriptionally induced by activation of nuclear receptors. However, this mechanism does not account for the increased CYP epoxygenase levels with a BOR diet since mRNA levels were unaltered. Likewise, mRNA stability changes can not be responsible for the changes in CYP epoxygenase levels. Post-transcriptional modifications of protein translation or stabilization are known to be important in regulating CYP expression (Song et al., 1989). Particularly, CYP2C23 protein levels were upregulated during dietary salt loading, while the mRNA levels remain unchanged (Holla et al., 1999). Similar observations published recently also demonstrated an increase in CYP2C23 protein levels with no change in mRNA levels in transgenic rats

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overexpressing renin and angiotensinogen genes (Muller et al., 2004). It is reasonable to hypothesize that a GLA-rich diet induces CYP epoxygenase protein levels by stabilizing the protein and/or decreasing proteolysis.

In summary, these studies demonstrate that a GLA-enriched diet induces CYP2C and CYP2J arachidonic acid epoxygenases and endogenous EET levels in the SHR renal cortex. The antihypertensive properties of EETs suggest that GLA-induced changes in arachidonic acid metabolism contribute to the reduction of blood pressure in BOR-fed SHRs. A similar association between arachidonic acid metabolism and blood pressure regulation should be explored in humans on a GLA-enriched diet.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Renal microsomal arachidonic acid metabolism in SHRs fed a BOR or SES diet. A) CYP eicosanoid formation in incubations of renal cortical microsomes from SES (solid bar) and BOR (hatched bar) treated SHRs with [1-^{14}C]-arachidonic acid. The sum of EET and DHET formation represents epoxygenase activity. B) Regioselective EET formation was measured in incubations of arachidonic acid with renal cortical microsomes from SES (solid bars) and BOR (hatched bars) samples. Metabolites were extracted from the incubation mixture and quantified by HPLC with radiometric detection. Values shown are the mean ± SEM of 5 samples/treatment group. *Significantly different from SES group, $p < 0.05$.

Figure 2. Endogenous DHET (A) and EET (B) levels in kidneys of SES- (solid bars) and BOR-fed (hatched bars) SHRs. Lipids were extracted from kidney cortex and quantified by LC/MS/MS as described in the Methods. The values shown are the mean ± SEM for 5 animals per group. **Significantly different from SES group, $p < 0.01$.

Figure 3. Western immunoblots of CYP arachidonic acid epoxygenases and soluble epoxide hydrolase in kidney cortex from BOR- and SES-fed SHRs. (A) Renal cortical microsomal proteins (10 µg) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and blotted with antisera against human CYP2J2, rat CYP2C11, rat CYP2C23, rat CYP4A1, mouse sEH or actin. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System. The blots shown are representative of results from four to six animals per experimental group. Actin was also detected on each blot and representative results from actin

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staining are shown. (B) The CYP and sEH protein levels were quantitated using the Odyssey Infrared Imaging System and expressed relative to the level of actin. Protein levels in samples from the SES-fed SHRs are set to 100%. *Significantly different from SES group, $p < 0.05$.

Figure 4. RNase protection assays of CYP epoxygenases and epoxide hydrolases in kidneys from SES- and BOR-fed SHRs. Total cortical RNA was used for RNase protection assays. RNA was hybridized with a CYP2C23 (A), CYP2E1 (B), CYP2J3 (C), CYP2J4 (D), mEH (E) or sEH (F) probe. In all cases, a rat GAPDH probe was included as an internal control. The autoradiograms are representative of five animals per experimental group. The top arrow indicates the CYP or EH transcript and the bottom arrow indicates the GAPDH transcript. Autoradiograms were visualized with a PhosphorImager and analyzed using ImageQuant software. There were no differences in the renal mRNA level of the CYP epoxygenases or epoxide hydrolase genes between the SES- and BOR-fed SHRs.

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Table 1

Arachidonic Acid Metabolism in Renal Cortical Microsomes from Sesame and Borage Oil Treated SHRs

	Metabolite Formation (% of Total)[a]	
Metabolite	**Sesame Oil**	**Borage Oil**
19-HETE	15.0 ± 0.61	10.9 ± 0.26**
20-HETE	50.8 ± 3.66	31.4 ± 0.90**
EETs	34.2 ± 3.94	57.7 ± 1.12**

[a]The values shown are the mean ± SEM of 5 samples per treatment group.

**Significantly different from sesame oil group, $p<0.01$.

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Figure 1

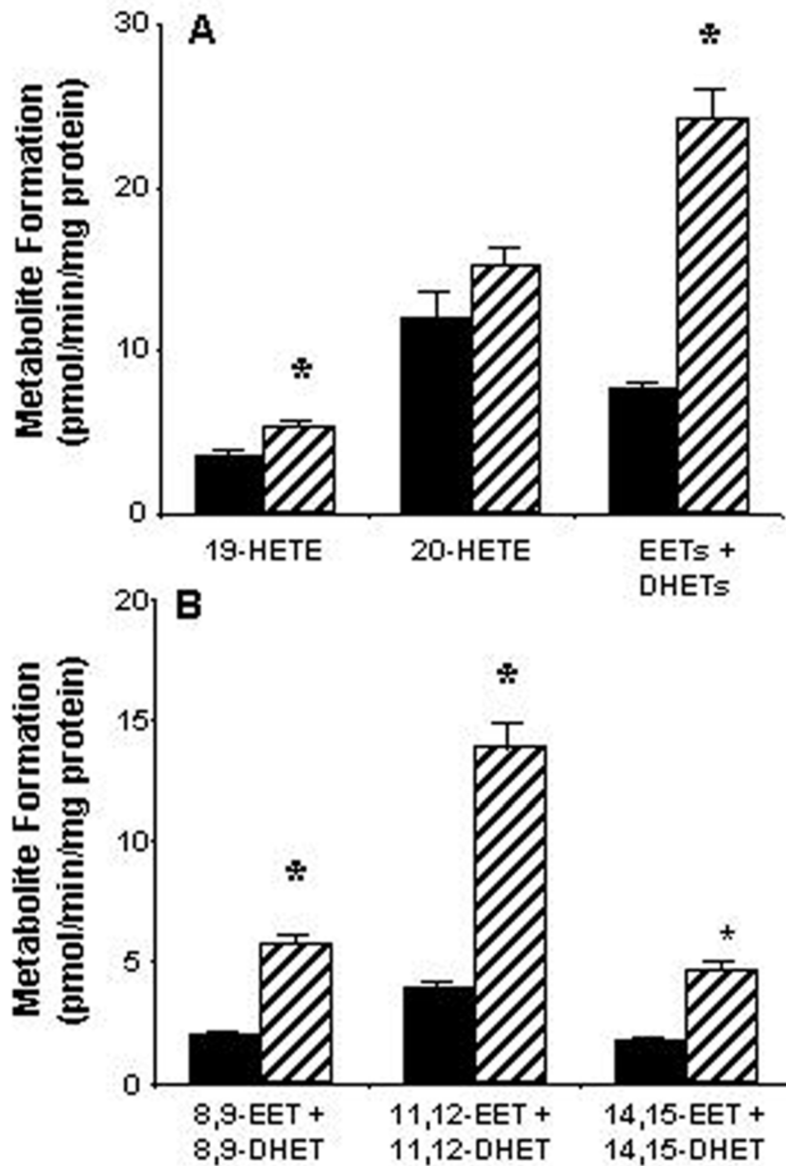

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Figure 2

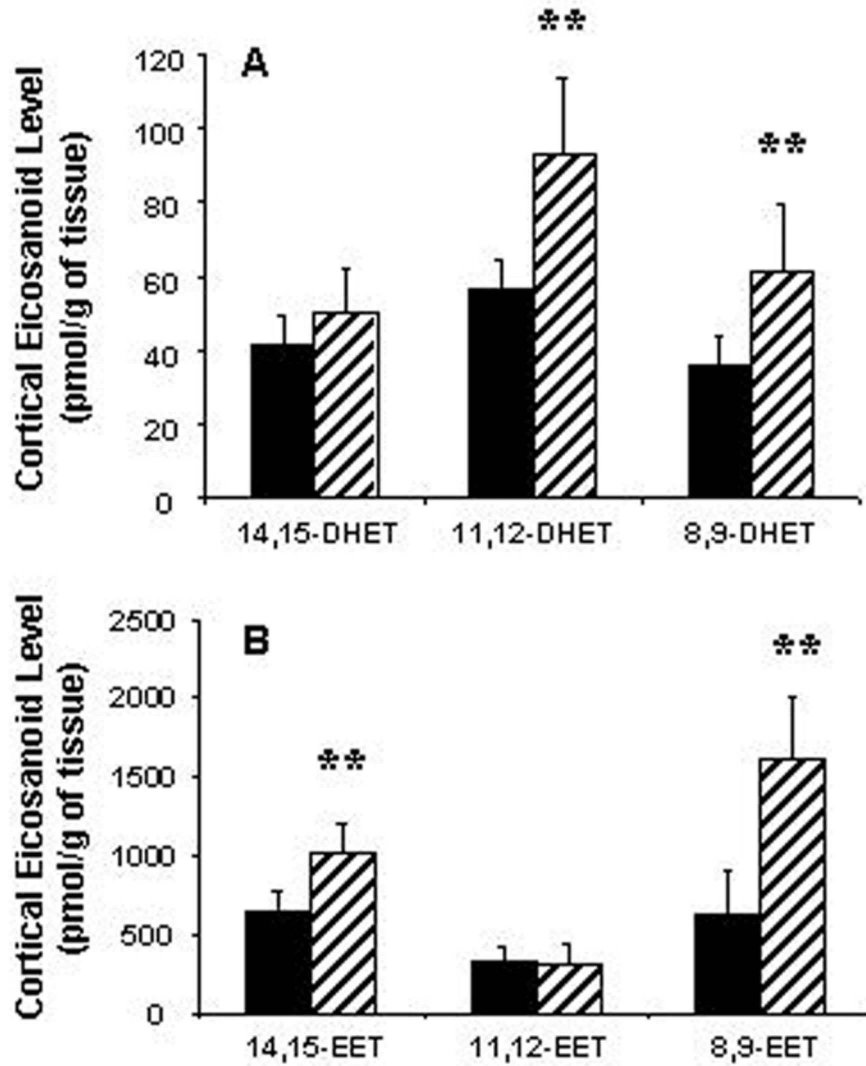

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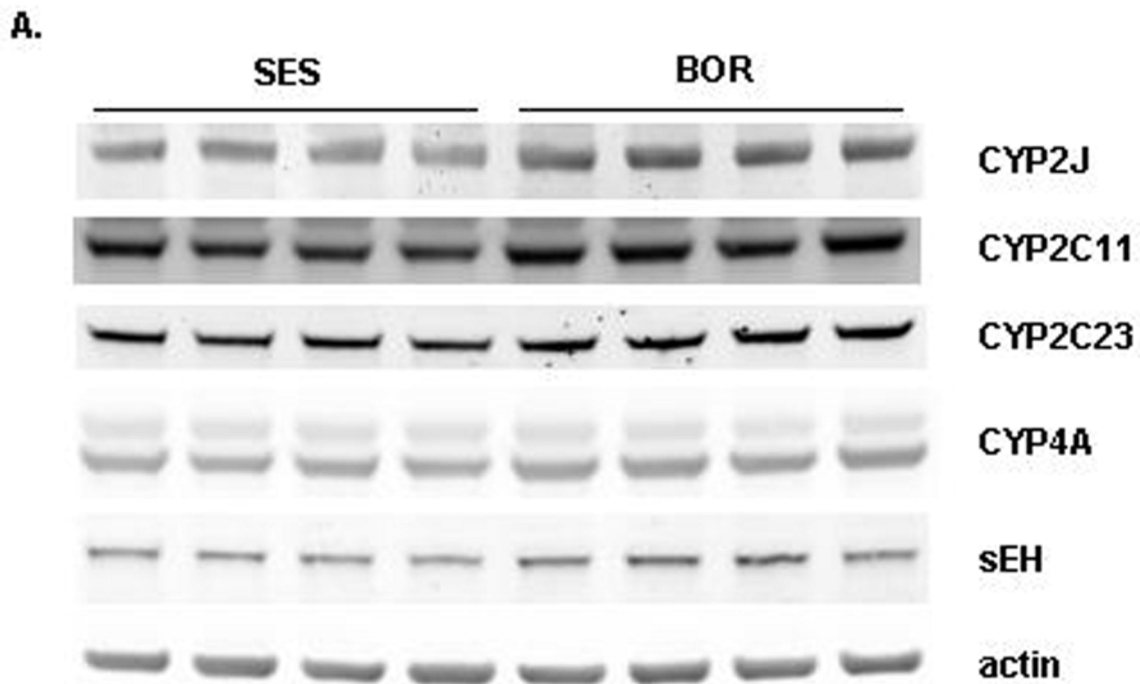
Figure 3



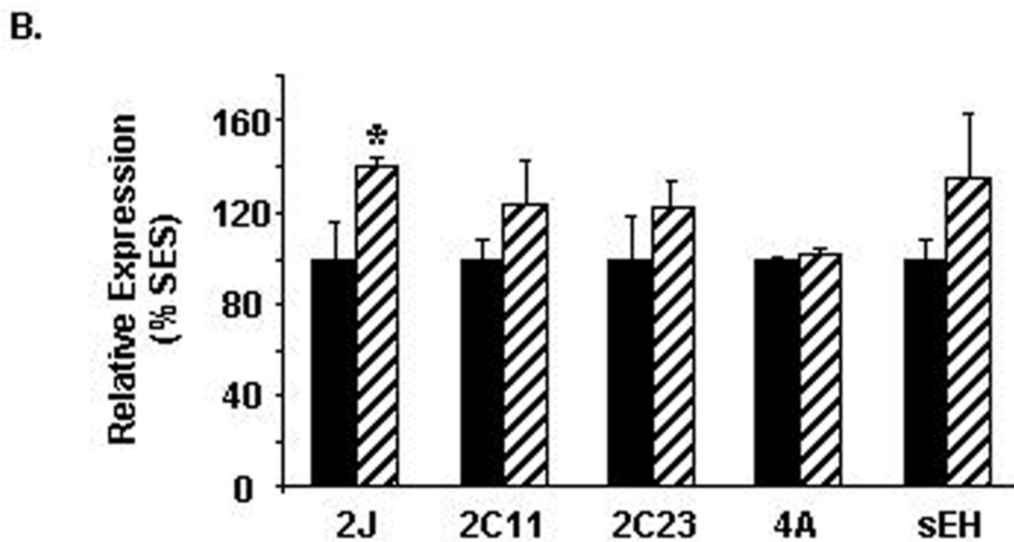


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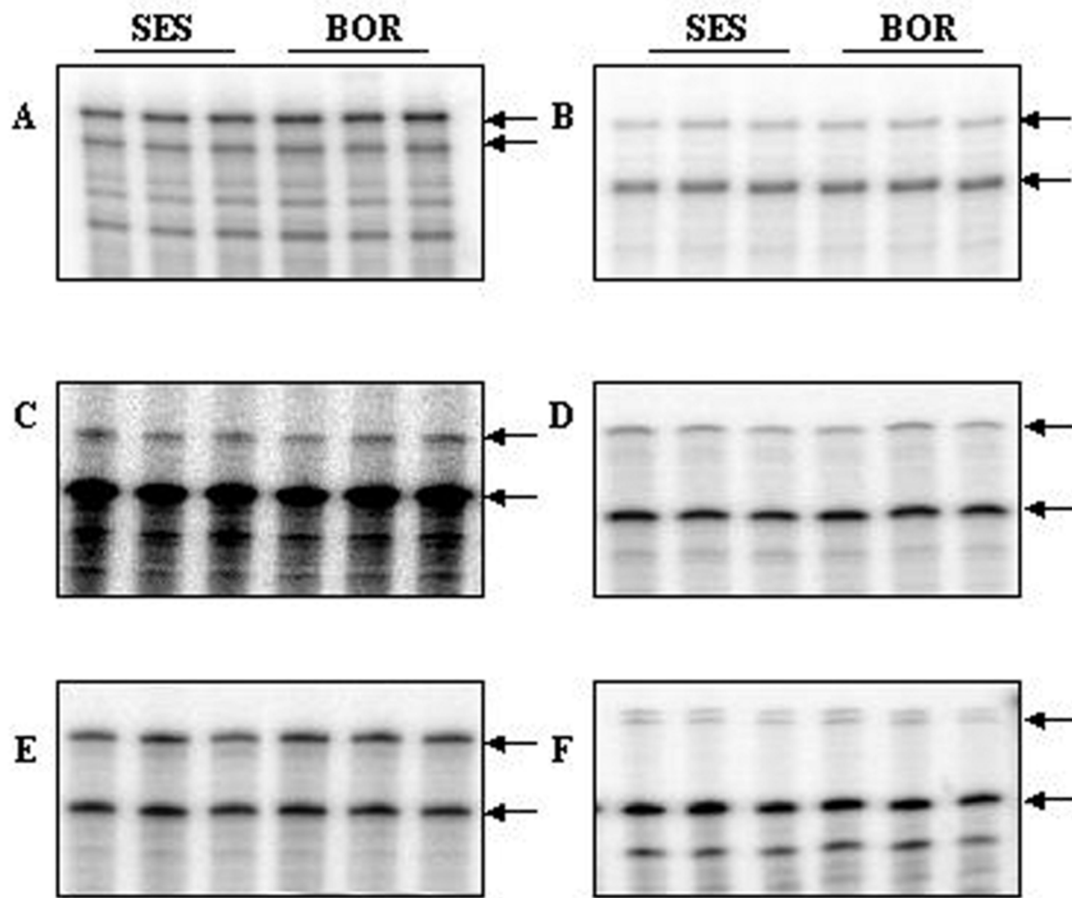
Figure 4



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