Docosahexaenoic Acid is an Antihypertensive Nutrient That Affects Aldosterone Production in SHR (44381)

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Abstract. **The effects of dietary docosahexaenoic acid (DHA), an ω-3 polyunsaturated fatty acid, on blood pressure and some pressure-regulating systems were measured in young spontaneously hypertensive rats (SHR). Plasma aldosterone and corticosterone levels, adrenal aldosterone production *in vitro*, and characteristics of adrenal angiotensin receptors were measured after 6 weeks of diet. Renal cytochrome P450 (CYP) 4A gene expression and arachidonic acid metabolism by renal microsomes were also investigated. Plasma cholesterol, triglycerides, and high-density lipoprotein cholesterol were measured. Diets contained either corn/soybean oil alone (CSO), or oil enriched with DHA. After 6 weeks, rats fed DHA had systolic blood pressures averaging 34 mmHg less than controls ($P < 0.001$). Plasma aldosterone levels were 33% lower in the DHA-fed animals than in controls (22 ± 3 vs. 33 ± 3.7 ng/dl, $P < 0.05$). Plasma levels of corticosterone were 18% lower in animals fed DHA than in controls, but this difference was not statistically significant. Adrenal glomerulosa cells from DHA-fed rats produced less aldosterone *in vitro* in response to angiotensin II, ACTH, or potassium. The difference was less marked when aldosterone production was stimulated by supplying exogenous corticosterone, suggesting an effect of DHA on postreceptor steps in signal transduction or the early pathway of aldosteronogenesis. We found no significant differences in angiotensin receptor subtype, number, or affinity. Production of arachidonic epoxides by renal microsomes was 17% lower in DHA-fed animals than in controls ($P < 0.05$). Renal cortical mRNA levels of CYP4A genes and formation of 19- and 20-hydroxyeicosatetraenoic acid (HETE) did not differ between dietary groups. Plasma total cholesterol and high-density-lipoprotein (HDL) levels were significantly reduced in SHR fed the DHA supplement, but triglyceride levels were not significantly different. The effects of DHA on steroid and eicosanoid metabolism may be part of the mechanism by which this fatty acid prevents some of the hypertension in growing SHR.** [P.S.E.B.M. 1999, Vol 221]

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Several studies have demonstrated an antihypertensive effect of dietary fish oil in hypertensive subjects (1–6) and spontaneously hypertensive rats (SHR) (7–14). Although fish oil is rich in two ω-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), some published results indicate that DHA alone can prevent hypertension (15, 16).

DHA's pressure-lowering mechanism is not known. Pertubations in the renin-angiotensin-aldosterone system or metabolism of arachidonic acid may be involved. We re-

cently demonstrated that a diet supplemented with borage oil, rich in γ-linolenic acid, an ω-6 polyunsaturated fatty acid, reduced the rate of increase of blood pressure in growing SHR. Adrenal glands from the borage oil–fed rats demonstrated reduced aldosterone production in response to angiotensin II, and reduced density and affinity of angiotensin receptors (17).

Several lines of evidence support a role for eicosanoids produced by cytochrome P450 (CYP) in the regulation of renal function and blood pressure. Both 20-hydroxyeicosatetraenoic acid (HETE) and eicosatrienoic acids (EETs) have potent effects on renal vessels and ion transport (18–20).

Increased renal arachidonic acid ω-hydroxylation has been reported in SHR relative to age-matched Wistar Kyoto (WKY) rats (21, 22). Recently, we showed that increased expression of the CYP4A3 and CYP4A8 genes and CYP4A proteins account for the increased arachidonic acid ω and ω-1 hydroxylation in the young SHR kidney (22). This suggests that altered CYP4A expression in the prehypertensive SHR kidney contributes to changes in renal function in this model of hypertension. There is evidence that a diet rich in polyunsaturated fatty acids can modulate the activity of CYP enzymes in the liver, so it is reasonable to postulate that dietary fats could affect those enzymes in the kidneys (23).

The purpose of the present study was to evaluate the effects of dietary DHA on blood pressure and some of its regulators in SHR. Systolic blood pressures were measured weekly during 6 weeks of dietary treatment with DHA. Plasma aldosterone, adrenal angiotensin receptors, and aldosterone production in adrenal glomerulosa cells were measured. To test the hypothesis that dietary administration of DHA modulates CYP4A genetic expression, CYP4A RNase protection assays were used. Arachidonic acid metabolism was measured directly in renal cortical microsomes.

Materials and Methods

Animals and Diet Preparation. Male, 7-week-old SHR were obtained from Harlan (Indianapolis, IN) and housed two to three per cage at constant temperature(26°C), humidity (60%), and lighting (12:12-hr light:dark cycle). The rats were randomly divided into two groups of 10–11 rats and were fed two special diets for 6 weeks. The diets consisted of a purified fat-free basal mix (Research Diets, Inc., New Brunswick, NJ) to which was added corn and soybean oils (CSO) or oil enriched with docosahexaenoic acid (DHA [DHASCO® from Martek Biosciences Corp., Columbia, MD]) (Table I). DHASCO® is derived from marine microalgae and has a high degree of oxidative stability compared to fish oil. The diets were designed to include similar proportions of saturated, monounsaturated, and polyunsaturated fatty acids while varying the content of docosahexaenoic acid(22:6n-3) (Table II).

Table I. Composition of Experimental Diets

Ingredient	Diet (g %)	
	CSO	DHA
Casein	150	150
DL-Methionine	2.3	2.3
Sucrose	231	231
Corn starch	423	423
Maltodextrin 10	40	40
Cellulose	50	50
Vitamin mix[a]	10	10
Mineral mix[a]	23	23
Calcium phosphate	13	13
Calcium carbonate	5.5	5.5
Corn oil	25	15
Soybean oil	25	15
DHA (DHASCO)	0	20

[a] Vitamin mix (Research Diets, V10001), Mineral mix (Research Diets, S10001A).

Table II. Fatty Acid Composition of Diets[a]

Fatty Acid	CSO	DHA
12:0	22.26	21.58
14:0	0.56	3.32
16:0	7.73	7.54
18:0	1.95	1.59
18:1n-9	13.51	14.71
18:1n-7	0.65	0.47
18:2n-6	33.07	21.36
18:3n-3	2.85	1.83
20:4n-6	—[b]	—
20:5n-3	—	—
22:6n-3	—	8.81

[a] Values are % of total fatty acids.
[b] Fatty acids were not detected.

The diets were stored at 0°C and provided fresh daily. Body weights were measured weekly, and heart weights were determined at the end of the study. The study was conducted in accordance with the guidelines of the Committee on Animal Research at the University of California, San Francisco.

Blood Pressure Measurement. Systolic blood pressure was measured at room temperature by a photoelectric tail cuff system (Model 179, IITC, Inc., Woodland Hills, CA). Blood pressure measurements were taken prior to the dietary treatments and weekly thereafter. Blood pressures are presented as the average of three separate readings.

Measurement of Plasma Aldosterone, Adrenal Aldosterone Production, and Angiotensin Receptors. Animals were sacrificed, alternating between control and experimental, under anesthesia with nitrous oxide(30%) and halothane(5%). Blood was obtained by cardiac puncture, plasma separated, and aldosterone measured by radioimmunoassay using a kit (Diagnostic Products Corp., Los Angeles, CA). Adrenal glands were excised and trimmed of fat. Adrenal capsules were obtained by incising each gland and expressing the bulk of the cortex and medulla under

gentle finger pressure. Zona glomerulosa cells adhering to the capsule were suspended by collagenase digestion as described (24). Cells were counted in a hemocytometer (Reichert Scientific Instruments, Buffalo, NY), and the proportion of glomerulosa cells estimated by their distinctive size. Glomerulosa cells constituted no less than 90% of the total in each preparation. Suspended cells were divided into aliquots and incubated with and without secretagogs. After 2 hr, the suspensions were centrifuged, and the aldosterone secreted into the medium was measured by radioimmunoassay using antibody from ICN Biomedicals, Inc. (Costa Mesa, CA) and labeled steroid (Diagnostic Products Corp.).

Aliquots of cells from the same batches as those used for aldosterone production were frozen and saved for measurement of angiotensin receptors. Thawed pellets were disrupted in a Potter-Elvejhem homogenizer (Koutes Scientific Glassware, Vineland, NJ), and the homogenate fractionated by centrifugation. The membrane fraction sedimenting between 1,000 and 27,000*g* was used for receptor measurements. Radioiodinated sarcosine[1]-angiotensin II was the radioligand, and graded amounts of unlabeled peptide were used to construct a dilution curve, which was analyzed by the method of Scatchard (25). Subtypes of angiotensin receptors were identified by adding either losartan or PD-123319 (gift from Parke-Davis, Ann Arbor, MI) to the binding assays to inhibit subtype 1 or 2, respectively.

Measurement of CYP4A Expression and Arachidonic Acid Metabolism. 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). Arachidonic acid was purchased from Nu Chek Prep (Elysian, MN). Protein was measured with the Pierce BCA protein assay from Pierce (Rockford, IL). All other reagents were of the highest grade available and were purchased from Fisher Scientific or Sigma Chemical Company.

For ribonuclease protection assays, kidneys were removed from anesthetized rats, dissected into the cortex, outer medulla, and inner medulla, and quickly frozen in liquid nitrogen. All tissue was stored at –80°C until preparation of RNA or microsomal samples. Total RNA was isolated from frozen renal cortex tissue by acid-phenol extraction. Construction of specific CYP4A riboprobes and details of the ribonuclease protection assays were described previously (22). The CYP4A3 probe was used to detect both CYP4A3 and CYP4A2 mRNA transcripts. The protected fragments spanned the following regions of the cDNA sequence: 1285–1557 nt of CYP4A1, 342–549 nt of CYP4A2, 214–526 nt of CYP4A3, 1300–1563 nt of CYP4A8, and 2–250 nt of GAPDH. Autoradiographs were scanned with an Ultro Scan XL laser densitometer from Pharmacia LKB (Piscataway, NJ), and the level of a given CYP4A mRNA was expressed relative to the level of GAPDH mRNA.

For assessment of renal arachidonic acid metabolism, microsomes were prepared from frozen cortex, using differential centrifugation, and stored at –80°C (26). Arachidonic acid metabolism was measured in cortical microsomes at a final substrate concentration of 0.01 m*M*. Reaction conditions, metabolite extraction, and separation and quantitation of arachidonic acid metabolites by reverse phase HPLC with radiometric detection were described previously (22).

Measurement of Plasma Lipids. Blood was drawn from anesthetized animals by cardiac puncture into EDTA-primed syringes. Plasma was separated by centrifugation at 800 rpm for 20 min at room temperature and stored at –70°C until assayed. Plasma samples were assayed for cholesterol, triglycerides, and high-density lipoproteins using a Hitachi 747 (Boehringer, Indianapolis, IN) in the Department of Clinical Chemistry, University of California, San Francisco.

Statistical Analyses. Values are expressed as means ± SEM. Statistical significance was determined with the Crunch statistical package (Crunch Software, Oakland, CA), using Student's *t* test for unpaired data. RNase protection assays and determination of arachidonic acid metabolism were performed in duplicate on RNA or protein samples from individual animals. The results are expressed as means ± SEM of five animals per treatment group. Statistical significance of differences between treatment groups was evaluated by an analysis of variance with posthoc multiple comparison testing with a modified *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Body and Heart Weight. Body weights were not significantly different between dietary groups at any time during the dietary treatments (Table III). There was also no significant difference between groups in heart weights obtained at the end of the study.

Blood Pressure. Systolic blood pressure was significantly lower in DHA-fed SHR compared to CSO-fed rats at 4, 5, and 6 weeks (Fig. 1). The DHA-enriched diet

Table III. Body and Heart Weights of SHR Fed a CSO- or DHA-Enriched Diet for 6 Weeks[a]

	CSO	DHA
Body Weight (g)		
Pre-diet	133 ± 6	121 ± 4
Week 1	216 ± 5	204 ± 4
Week 2	243 ± 6	235 ± 4
Week 3	264 ± 6	253 ± 5
Week 4	275 ± 6	276 ± 4
Week 5	289 ± 7	289 ± 4
Week 6	301 ± 8	297 ± 4
Heart weight (g)	1.1 ± 0.03	1.1 ± 0.02

[a] Values are means ± SEM; n = 10–11 rats per group.

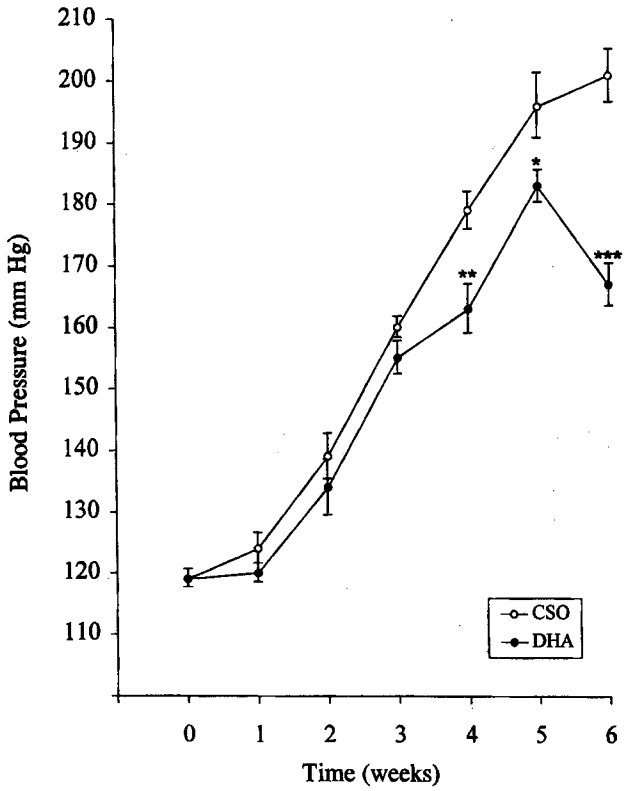

Figure 1. Effects of a DHA-enriched diet, compared to control (CSO), upon systolic blood pressure in SHR. Values are expressed as mean ± SEM of 10–11 rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. CSO diet.

reduced mean blood pressure at Week 6 by an average of 34 mmHg.

Plasma Aldosterone and Corticosterone. Aldosterone levels in plasma drawn while animals were anesthetized were 33% lower in rats fed the DHA supplement than in controls (21.98 ± 3.09 vs. 32.97 ± 3.30 ng/dl). This difference was significant by ANOVA ($P = 0.036$) or two-tailed t test ($P = 0.035$). Corticosterone in the same samples was 18% less in rats fed the DHA supplement than in controls (294.9 ± 26.1 vs. 359.7 ± 20.6 ng/ml), but this difference was not statistically significant ($P = 0.064$).

Adrenal Aldosterone Production and Angiotensin Receptors. Adrenal glomerulosa cells from DHA-fed rats produced less aldosterone in response to angiotensin II than cells from CSO-fed rats. The results of one experiment are shown in Figure 2. This result was similar to that of a second experiment done on the preceding day on a different batch of rats from the same cohort. The decrement in aldosterone production caused by DHA feeding was also seen when the stimulus *in vitro* was potassium or ACTH, but was much less evident when aldosterone production was stimulated by adding the precursor corticosterone, as shown by the bars in Figure 2.

Angiotensin receptors in both groups of rats were predominantly of the AT_1 subtype. Scatchard analysis of binding data on membranes from the same batch of adrenal cells showed no significant differences in receptor density or affinity between those from DHA-fed and control animals (data not shown).

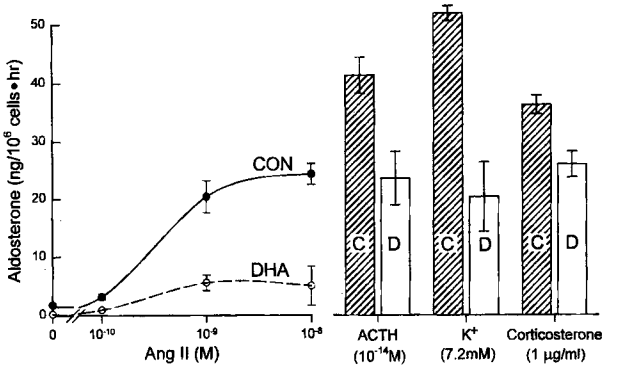

Figure 2. Aldosterone production by adrenal cells from rats fed a diet containing supplemental DHA or a diet containing corn oil/soybean oil (CON). The curves are responses to graded doses of angiotensin II, and the bars are responses to maximal doses of potassium, ACTH, or added corticosterone.

CYP4A Expression and Arachidonic Acid Metabolism. The cortical mRNA level of each of the renally expressed CYP4A genes was measured using gene-specific RNase protection assays previously developed in this laboratory (22). CYP4A mRNA levels were not altered in the DHA-fed animals (Fig. 3). This indicated that DHA and its metabolites were not acting as CYP4A modulators. Similar results were found when arachidonic acid hydroxylase activity was measured in renal cortical microsomes. Formation of both 19- and 20-HETE was unaltered in the DHA-treated kidneys relative to the control-fed animals (Table IV), consistent with the lack of effect of the DHA-enriched diet on CYP4A mRNA levels. By contrast, arachidonic acid

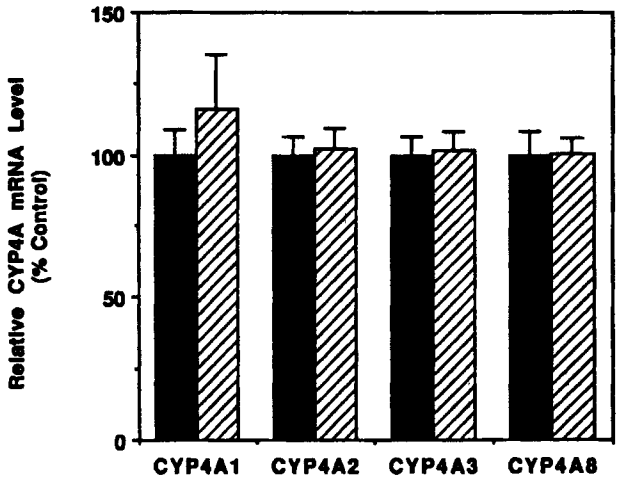

Figure 3. Effects of diet supplemented with DHA or control CSO on CYP4A mRNA levels. RNA was prepared from the renal cortex of individual rats, and CYP4A mRNA levels were quantitated using gene-specific RNase protection assays. CYP4A mRNA levels were normalized to the level of GAPDH mRNA in each sample and were expressed as a percentage of the value in the CSO-fed animals. Values shown are the mean ± SEM of CSO- (solid bars) and DHA- (striped bars) fed SHR ($n = 5$ per group). There were no significant differences in renal cortical CYP4A levels between the CSO or DHA-fed animals.

Table IV. Arachidonic Acid Metabolism by Renal Cortical Microsomes from SHR Rats Fed Either the CSO- or DHA-Enriched Diet for 6 Weeks[a]

Metabolite	Metabolite (pmol/min/mg protein)	
	CSO	DHA
19-HETE	4.61 ± 0.32	4.28 ± 0.43
20-HETE	26.2 ± 1.31	24.1 ± 2.62
Epoxides	11.1 ± 0.41	9.27 ± 0.34[b]

[a] Microsomes were prepared from the renal cortex and incubated with ^{14}C-arachidonic acid at 37°C. Metabolites were extracted and quantified by HPLC with radiometric detection. Values are means ± SEM, n = 5 rats per group. Epoxides are the sum of all regio- and isomeric epoxides and their corresponding dihydroxy-eicosatrienoic acid metabolites.
[b] Significant difference at $P < 0.05$ compared with the CSO diet.

epoxygenase activity was slightly decreased in the DHA-treated kidneys compared to controls.

Plasma Lipids. A significant reduction in plasma cholesterol and high-density lipoprotein cholesterol (HDL) was found in DHA-fed SHR compared to CSO-fed SHR (Table V).

Discussion

This study demonstrates that dietary DHA attenuates the development of hypertension in prehypertensive SHR by an average of 34 mmHg systolic following 6 weeks of feeding. These findings are remarkably similar to those of other investigators who reported a 35-mmHg difference in systolic blood pressure in SHRSP fed diets containing 5% DHA for 14 weeks, and a 33-mmHg difference in SHR fed 4.5% DHA-enriched diets for 12 weeks (15, 16).

One humoral support of blood pressure is the renin-angiotensin-aldosterone axis. In a previous experiment, we found that a diet rich in γ-linolenic acid attenuated the pressure elevation of young SHR and reduced the apparent responsiveness of the adrenal to angiotensin, as reflected in a lower ratio of plasma aldosterone to plasma renin activity (17). In this study, we found that plasma aldosterone levels were lower in DHA-fed animals than controls. Congruent with this observation, adrenal glomerulosa cells isolated from animals that were fed DHA produced less aldosterone than those from control animals fed corn and soybean oil when stimulated by each of the classical secretagogs (angiotensin, ACTH, and potassium). The difference between glands from DHA-fed and control animals was much smaller when aldosterone production was stimulated by adding excess substrate. These findings would be consistent with an effect of dietary DHA on a postreceptor step in signal transduction or the "early pathway" of aldosterone biosynthesis. Both of those sites would be circumvented by adding corticosterone. Among logical possibilities for DHA action are calcium mobilization, protein phosphorylation, or the labile proteins that facilitate cholesterol movement into adrenal mitochondria.

Table V. Plasma Lipids in SHR Rats Fed Either the CSO- or DHA-Enriched Diet for 6 Weeks

Lipid	CSO	DHA
Cholesterol (mg/dl)	78 ± 2[a]	58 ± 2[b]
Triglycerides (mg/dl)	223 ± 19	176 ± 19
HDL (mg/dl)	52 ± 3	42 ± 1[c]

[a] Values are means ± SEM, n = 10 rats per group.
[b] $P < 0.001$ compared with the CSO diet.
[c] Significant difference at $P < 0.05$.

In a previous experiment, we showed that a dietary supplement, borage oil, rich in γ-linolenic acid, reduced adrenal angiotensin receptor number and affinity (17). In the present study, dietary DHA caused no significant change in angiotensin receptors. This is consistent with the observed effects on other stimuli.

It is interesting to note that the blood pressure lowering effect of DHA is similar to that of the 18-carbon ω-6 polyunsaturated fatty acid, γ-linolenic acid. We have previously shown that dietary feeding for 7 weeks with 24% γ-linolenic acid in borage oil in 6-week-old SHR decreased blood pressure by 30 mmHg compared to controls (27). This effect was not attributed to changes in vascular reactivity since contractile and relaxation responses of the thoracic aorta were unchanged. We have recently reported similar findings in 7-week-old SHR fed a DHA-enriched diet for 6 weeks (28). No significant differences were found between DHA and control SHR in the contractile responses of aortic vessels to potassium chloride, norepinephrine, angiotensin II, or the vasorelaxant responses to acetylcholine, sodium nitroprusside, papaverine, methoxyverapamil, and TMB-8. Therefore, the antihypertensive properties of γ-linolenic acid and DHA are not mediated by alterations in vascular responses.

The relevance of the effects exerted by dietary DHA on adrenal cells to its effect on blood pressure is difficult to assess. In a review of the effects of fish oil on blood pressure, Kenny and Egan concluded that the hypotensive effect of fish oil was most evident when blood pressure was dependent on the renin-angiotensin-aldosterone system, as in salt-deprivation (29). Our diets were not low in sodium chloride content, but blood pressure in chow-fed SHR can be lowered by administration of drugs that interrupt the renin-angiotensin-aldosterone axis, so the effect we observed on the adrenal may well have affected blood pressure (30).

It has been proposed that the hypotensive properties of fish oil may result from changes in eicosanoid metabolism (13). Vascular smooth muscle and endothelial cells have been shown to incorporate ω-3 polyunsaturated fatty acids at the expense of ω-6 polyunsaturated fatty acids (31, 32). The subsequent reduction in arachidonic acid limits endogenous synthesis of vasoactive eicosanoids such as the potent vasoconstrictor TXA_2 (14). Arachidonic acid is also the precursor for cytochrome P450–derived eicosanoids, hydroxyeicosatetraenoic (HETE) and epoxyeicosatrienoic (EET) acids (18). These metabolites have powerful vasoac-

tive properties due to their effects on cell membrane ion permeability and fluxes (18–20).

We found no effect of dietary DHA on renal cortical CYP4A expression or 20-HETE formation. This differs from results of an experiment reported recently in which Sprague-Dawley rats fed diets enriched with fish oil, EPA, or DHA for 5 days had increased levels of hepatic CYP4A2 mRNA (23). The two studies differed in duration of dietary treatment and in the age and strain of rat.

Since increased intake of ω-3 polyunsaturated fatty acids has been associated with decreased levels of arachidonic acid incorporation into the phospholipid pool of vascular smooth muscle and endothelium, we speculated that this would lead to decreased formation of arachidonic acid metabolites in the kidney (31, 32). We observed a slight decrease in arachidonic acid epoxygenase activity in renal cortical microsomes from DHA-fed rats. Renal arachidonic acid epoxide formation is catalyzed by CYP2C23, CYP2E1, and CYP2J3 (33–35). The activity of one or more of these isoforms may be altered by DHA feeding and the reduction in arachidonic acid availability. EETs and their corresponding dihydroxy derivatives (DHETs) have vasoactive properties and can modulate intracellular ion concentrations throughout the nephron (19, 20). In general, the EETs and DHETs are considered antihypertensive, so the small decrease in their formation in the kidneys of DHA-fed SHR rats seems unlikely to account for the decrease in blood pressure in these animals.

Previous human and rat studies have shown that fish oils lower plasma triglycerides (36, 37). We found no significant effect of DHA on triglycerides, consistent with another investigation that found no effect of DHA in rats (38). DHA alone does not influence hepatic synthesis or release of triglycerides. We did observe a significant decrease in plasma cholesterol and HDL in SHR fed the DHA-enriched diet. This has also been reported in SHR fed a fish oil diet (39). Several mechanisms may contribute to the hypocholesterolemic effect of DHA, including changes in lipoprotein receptors (40). To our knowledge, this is the first study to document an HDL-lowering effect of a DHA-enriched diet in SHR. HDL is the major cholesterol-carrying lipoprotein in rats. Since a portion of HDL is derived from the conversion of VLDL and chylomicrons to LDL, it is possible that the reduction in HDL levels is a reflection of decreased circulating VLDL and chylomicrons. However, further research is needed to investigate this mechanism.

In summary, we have demonstrated that dietary DHA attenuates the development of hypertension in prehypertensive SHR. The effect may be mediated by blunting of the renin-angiotensin-aldosterone system at the level of adrenal synthesis of aldosterone, or by changes in arachidonic acid metabolism. Dietary DHA also decreased plasma total and HDL cholesterol but not triglycerides. We can only speculate about the relevance of diminished aldosterone production or altered arachidonic acid metabolism to the lower arterial pressure of SHR fed DHA, but the data are consistent with their involvement.

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