

PPAR α Activator Effects on Ang II–Induced Vascular Oxidative Stress and Inflammation

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Abstract—Docosahexaenoic acid (DHA), a peroxisome proliferator–activated receptor- α (PPAR α) activator, reduces blood pressure (BP) in some hypertensive models by unclear mechanisms. We tested the hypothesis that DHA would prevent BP elevation and improve vascular dysfunction in angiotensin (Ang) II–infused rats by modulating of NADPH oxidase activity and inflammation in vascular wall. Sprague-Dawley rats received Ang II (120 ng/kg per minute SC) with or without DHA (2.5 mL of oil containing 40% DHA/d PO) for 7 days. Systolic BP (mm Hg), elevated in Ang II–infused rats (172 ± 3) versus controls (108 ± 2 , $P<0.01$), was reduced by DHA (112 ± 4). In mesenteric small arteries studied in a pressurized myograph, media/lumen ratio was increased ($P<0.05$) and acetylcholine-induced relaxation impaired in Ang II–infused rats ($P<0.05$); both were normalized by DHA. In blood vessels of Ang II–infused rats, NADPH oxidase activity measured by chemiluminescence and expression of adhesion molecules intercellular adhesion molecule and vascular cell adhesion molecule-1 were significantly increased. These changes were abrogated by DHA. PPAR α activator DHA attenuated the development of hypertension, corrected structural abnormalities, and improved endothelial dysfunction induced by Ang II. These effects are associated with decreased oxidative stress and inflammation in the vascular wall. (*Hypertension*. 2002;40:866-871.)

Key Words: hypertension, experimental ■ resistance ■ remodeling ■ muscle, smooth, vascular ■ free radicals

Hypertension is a well-established risk factor for the development of atherosclerosis. Angiotensin (Ang) II, the final mediator of the renin-angiotensin system, plays a major role in hypertension.¹ Ang II–induced hypertension exhibits vascular structure abnormalities (vascular remodeling) and endothelial dysfunction. Vascular remodeling consists of many processes such as vascular smooth muscle cell (VSMC) growth, apoptosis, low-grade inflammation, and vascular fibrosis.² In addition, there is increasing evidence that Ang II through activation of AT₁ receptors increases generation of reactive oxygen species (ROS) in the vessel wall,³ mainly through activation of membrane-bound NADH/NADPH oxidase in vascular cells.^{3,4} Furthermore, Ang II increases redox-sensitive and proinflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule (ICAM), which play a critical role in the initiation and progression of atherosclerosis.^{5,6}

Docosahexaenoic acid (DHA), an ω -3 polyunsaturated fatty acid, has been reported to lower blood pressure (BP) and prevent the development of hypertension in experimental models as well as in humans.^{7–9} DHA is also recognized as an activator of peroxisome proliferator-activated receptor (PPAR)- α , which belongs to the nuclear

hormone receptor superfamily and plays an important role in lipid metabolism. Apparently, PPAR α is implicated in metabolic disorders such as dyslipidemia, which is one of the risk factors of atherosclerosis. In addition to the heart and the liver, in which fatty acid metabolism is very active, the endothelium,¹⁰ in VSMC,¹¹ and monocytes and monocyte-derived macrophages¹² express PPAR α . This has suggested that PPAR α may exert direct beneficial effects on the vascular wall.¹³ Indeed, PPAR α activation reduces expression of monocytic and proinflammatory genes.^{11,14} Moreover, PPAR α activators inhibit inducible nitric oxide synthase in macrophages and prevent the IL-1–induced expression of IL-6 and cyclooxygenase-2 as well as thrombin-induced endothelin-1 expression as a result of a negative transcriptional regulation of nuclear factor-kappa B (NF κ B) and activator protein-1 signaling pathways, indicating a novel role for PPAR α in vascular endothelial function.^{10,15} PPAR α activation also inhibits VSMC proliferation and induces apoptosis in human macrophages and VSMCs.^{12,16,17} The pleiotropic effects of PPAR α activators on the plasma lipid profile and vascular wall inflammation may contribute to the inhibition of atherogenesis.

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In this study, we tested the hypothesis that the PPAR α activator DHA would prevent BP elevation and improve endothelial dysfunction in Ang II-induced hypertension by modulating NADPH oxidase activity and inflammation in vascular wall.

Methods

Animal Experiments

The study was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and followed guidelines of the Canadian Council for Animal Care. Male Sprague-Dawley rats (weight 200 g) were infused subcutaneously with Alzet osmotic minipumps (Alza Corp) with Ile⁵-Ang II (Peninsula) at a dose of 120 ng/kg per minute for 7 days and/or treated with DHA (2.5 mL of oil containing 40% DHA per day PO, Martek Biosciences Co). Systolic BP was measured by the tail-cuff method. Rats not fasted overnight were killed by decapitation. The mesenteric bed was dissected; one segment was used for preparation of small arteries and confocal immunohistochemical evaluation. A portion of aorta was fixed in 10% buffered formalin solution and embedded in paraffin. Tissue sections (7 μ m thick) were prepared for collagen staining and immunohistochemical analysis. Another portion of aorta was used to measure NADPH oxidase activity.

Preparation and Study of Small Arteries

Third-order superior mesenteric arteries (length \approx 2 mm) were dissected and placed in cold physiological salt solution. They were mounted on glass microcannulas in a pressurized myograph. Intraluminal pressure was set to 45 mm Hg with a servo-controlled pump. Endothelium-dependent relaxation was assessed with cumulative doses of acetylcholine (10^{-9} to 10^{-4} mol/L) after precontraction with 10^{-5} mol/L norepinephrine. Endothelium-independent relaxation was assessed with cumulative doses of sodium nitroprusside (10^{-8} to 10^{-3} mol/L). Vessels were then deactivated by perfusion with Ca²⁺-free physiological salt solution containing 10 mmol/L EGTA for 30 minutes. Lumen and media were measured with intraluminal pressure at 45 mm Hg. Media cross-sectional area was calculated as $[(\pi/4) \times (D_e^2 - D_i^2)]$, where D_e and D_i are the external and lumen diameters, respectively.

NADPH Oxidase Activity

Freshly excised thoracic aorta was cleaned of adherent adipose tissue, and a segment was incubated in HEPES buffer at 37°C for 30 minutes. Activity of NADPH oxidase was measured with a luminescence assay, as previously described.⁴ Briefly, lucigenin (5 μ mol/L) was used as the electron acceptor and NADPH (100 μ mol/L) as the substrate. The reaction was started by the addition of NADPH to the tissue sample. Luminescence was measured every 1.8 seconds for 3 minutes in a luminometer (AutoLumat LB 953, Berthold). A buffer blank was subtracted from each reading. Activity was expressed as counts/min per milligram of dry tissue weight.

Confocal Immunohistochemical Assay for VCAM-1 in Mesenteric Arteries

The confocal immunohistochemical assay for VCAM-1 was performed as previously described.¹⁸ Superior mesenteric arteries were dissected and mounted on glass microcannulas in a pressurized myograph as described in the "Preparation and Study of Small Arteries" section. After the vessel had been equilibrated for 1 hour, sodium nitroprusside (10^{-3} mol/L) was added to the chamber to relax the vessel. After 20 minutes, the vessel was fixed in 3.5% formaldehyde and 0.75% glutaraldehyde in 0.05 mol/L PBS, which has been shown to preserve artery structure with little background fluorescence, at pH 7.4 for 30 minutes. After washing 3 times with PBS, the sutures tied at the end of the artery were loosened and the artery was gently transferred from the chamber to a Petri dish and cut longitudinally in the middle. The artery was then permeabilized by 0.1% triton X-100 (pH 8.0) for 5 minutes. The vessel was exposed to 0.5% BSA/PBS and goat serum for 10 minutes at 42°C and then incubated with VCAM-1 antibody (Santa Cruz Biotechnology) at a dilution of 1:20 in PBS at 4°C overnight. After washing in PBS, the vessel was incubated 30 minutes with secondary antibody (Alexa fluor 647 goat anti-rabbit IgG) at a dilution of 1:100 at 37°C. After staining with Alexa Fluor rhodamin phalloidin, the vessel was mounted in glycerol:PBS (1:1). Fluorescence was detected with a Zeiss Axiovert 100 mol/L confocal microscope at a magnification of 63.

Immunohistochemical Assay for ICAM

Paraffin-embedded sections of aorta were rehydrated, and antigen retrieval was performed with 0.01 mol/L citrate buffer (pH 6.0) at 95°C for 20 minutes. Endogenous peroxidase activity was quenched by incubation in PBS containing 3% hydrogen peroxide. Sections were blocked with goat serum for 30 minutes, followed by incubation with anti-rat ICAM-1 antibody (Santa-Cruz) (1:100) for 1 hour at 37°C. Sections were washed with PBS and incubated with conjugated IgG secondary antibody (Vectastain ABC kit, Vector Laboratory) for 1 hour at room temperature. The signal was revealed with 3,3'-diaminobenzidine (Sigma). Sections were counterstained with hematoxylin and mounted. Negative controls were incubated with nonimmune rabbit IgG in place of primary antibody. Spleen was used for positive controls of ICAM.

Measurement of Plasma Renin Activity, Aldosterone, and Lipids

Plasma renin activity (PRA) was measured by radioimmunoassay of Ang I produced after a 2-hour incubation of plasma at 37°C and pH 6.5. Plasma aldosterone was measured with an ALDOCTK-2 (P2714) assay kit from DiaSorin. Plasma was assayed for total (free and esterified) cholesterol and triacylglycerol with a COBAS MIRA-S automated analyzer using enzymatic reagents (Hoffman-LaRoche). NEFA (nonesterified fatty acids) were measured by enzymatic colorimetric method (Wako NEFA C test kit, Wako Chemicals GmbH).

Body Weights, PRA, Aldosterone, Cholesterol, Triacylglycerol, and NEFA in Ang II-Infused Rats \pm DHA

Parameter	Control	Ang II	Ang II+DHA	DHA
Body weight, g	240 \pm 5	248 \pm 3	247 \pm 3	248 \pm 3
PRA, ng Ang I/mL per hour	3.5 \pm 0.6	0.7 \pm 0.4†	0.5 \pm 0.2†	3.1 \pm 0.3‡
Plasma aldosterone, pg/mL	146 \pm 35	245 \pm 100	300 \pm 91*	180 \pm 16
Plasma cholesterol, mmol/L	1.71 \pm 0.05	1.54 \pm 0.12	1.03 \pm 0.08*‡	1.06 \pm 0.08*‡
Plasma triacylglycerol, mmol/L	0.99 \pm 0.09	0.88 \pm 0.13	0.49 \pm 0.09	0.74 \pm 0.14
Plasma NEFA, mEq/L	0.27 \pm 0.04	0.25 \pm 0.03	0.28 \pm 0.04	0.33 \pm 0.04

Data are mean \pm SEM of 5 to 8 rats. * P <0.05, † P <0.01 vs control, ‡ P <0.05 vs Ang II group.

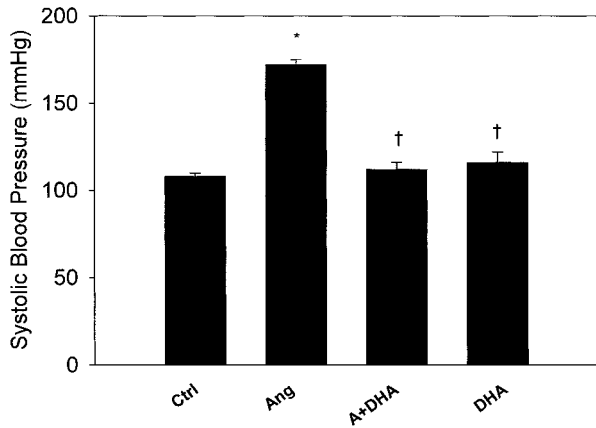


Figure 1. Systolic BP in Ang II-infused rats treated with or without DHA for 7 days. Results are mean±SEM (n=5 to 8 per group). * $P<0.05$ vs control, † $P<0.05$ vs Ang II group. Ctrl indicates control; DHA, docosahexaenoic acid; A+DHA, Ang II infusion+DHA.

Data Analysis

Values are presented as mean±SEM. Results were compared by 1-way ANOVA, followed by Tukey-Kramer's test. A value of $P<0.05$ was considered significant.

Results

Body Weight, BP, PRA, Aldosterone, and Lipids

After 7-day treatment, body weight was similar in all groups (Table). BP increase induced by Ang II infusion ($P<0.01$ versus control) was prevented by DHA (Figure 1). DHA did not affect BP in normal rats.

PRA was significantly reduced in Ang II-infused rats and unaffected by DHA (Table). Plasma aldosterone was increased in Ang II-infused rats with or without DHA. DHA alone had no effect on PRA or plasma aldosterone in control rats.

Ang II had no effect on plasma lipids (Table). DHA significantly decreased plasma cholesterol and slightly reduced plasma triacylglycerol in Ang II-infused and normal rats. However, it had no effect on NEFA concentrations.

Morphology and Endothelial Function of Resistance Arteries

Ang II infusion resulted in increased ($P<0.05$) media/lumen ratio of resistance arteries (Figure 2), which was normalized by treatment with DHA. Ang II-infused rats exhibited impaired acetylcholine-induced relaxation (Figure 3A), which improved ($P<0.05$) under DHA. Endothelium-independent relaxation by sodium nitroprusside was similar in all groups (Figure 3B). Relaxation to acetylcholine and sodium nitroprusside was unaffected by DHA alone.

NADPH Oxidase Activity in Aorta

NADPH oxidase activity in aorta was 2-fold increased in Ang II-infused rats compared with control rats (Figure 4). This increase was abrogated by DHA. Normotensive rats receiving DHA alone had normal NADPH oxidase activity.

Vascular Expression of Adhesion Molecules ICAM and VCAM-1

Expression of ICAM in the aorta of Ang II-infused rats was higher than in normotensive rats (Figure 5). Similarly, confocal microscopy showed significantly higher expression of VCAM-1 in the endothelium of mesenteric vessels from Ang II-infused rats, compared with control rats (Figure 6). These effects were abolished by DHA, whereas DHA alone had no effect on ICAM or VCAM-1 expression in control rats.

Discussion

In this study, we demonstrate that BP lowering by DHA is associated with improvement of endothelium-dependent vasodilation and vascular structure in Ang II-infused rats. We

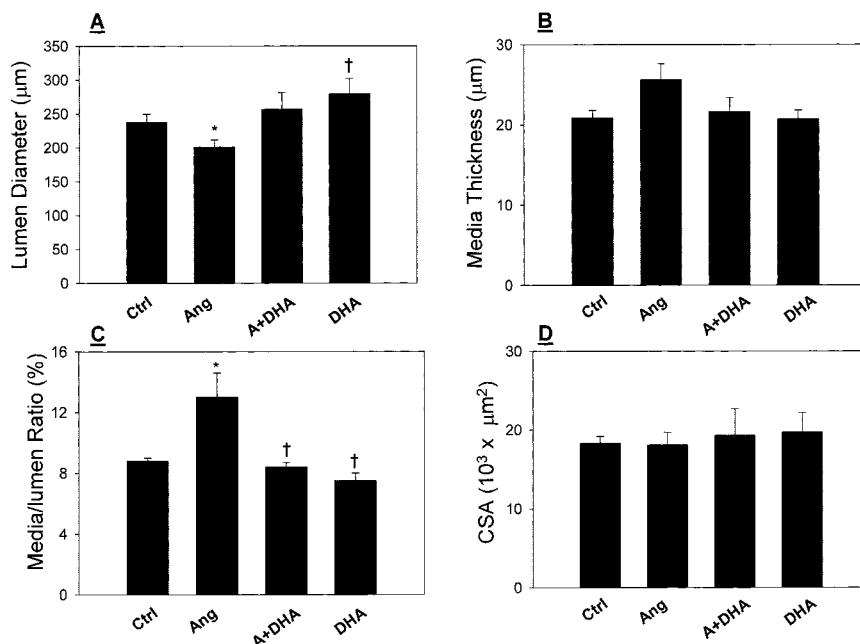


Figure 2. Lumen diameter (A), media thickness (B), media/lumen ratio (C), and cross-sectional area (CSA, D) of small mesenteric arteries of Ang II-infused rats treated with or without DHA for 7 days. Results are mean±SEM (n=5 to 8 per group). * $P<0.05$ vs control, † $P<0.05$ vs Ang II group.

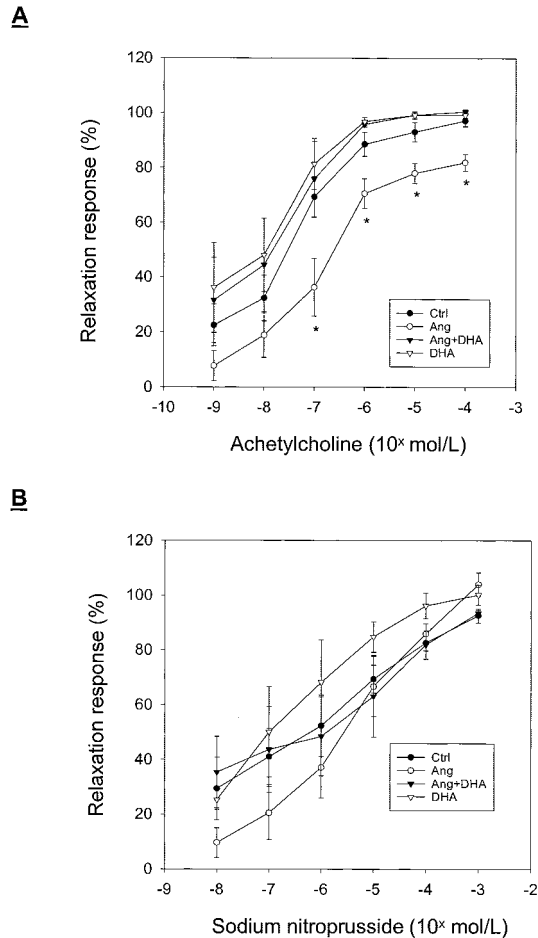


Figure 3. Endothelium-dependent relaxation in response to acetylcholine (A) and endothelium-independent relaxation in response to sodium nitroprusside (B) in small mesenteric arteries of Ang II-infused rats treated without or with DHA for 7 days. Results are mean \pm SEM (n=5 to 8 per group). Relaxation is expressed as percent increase in intraluminal diameter after precontraction with 10^{-5} mol/L norepinephrine. * $P < 0.05$ vs other groups.

also show that DHA exhibited antioxidant and anti-inflammatory effects on the vascular wall. These data suggest that the PPAR α activator DHA may have direct effects on blood vessels by modulating superoxide generation and inflammation.

Our findings agree with and extend recent data from Tabernero et al,¹⁹ who demonstrated that fenofibrate, also a PPAR α activator, improved endothelial nitric oxide-mediated vasodilation by increasing antioxidant activity of mouse blood vessels, suggesting a role of PPAR α in vascular wall. Since stimulation of AT₁ receptors by Ang II may lead to activation of membrane-bound vascular NADPH oxidase, which is a major source of ROS in vascular cells,^{4,20} mechanisms whereby DHA improved endothelial dysfunction of resistance arteries of Ang II-infused rats could include attenuation of Ang II-induced oxidative stress secondary to downregulation of AT₁ receptors, resulting in less nitric oxide degradation. We have demonstrated that DHA decreased NADPH oxidase activity in aorta of Ang II-infused rats. Whether this effect of DHA is mediated by AT₁ receptor

downregulation or not remains to be elucidated. Decreased BP could also contribute to improvement of endothelial dysfunction. However, we previously demonstrated that the vasodilator hydralazine decreased BP without any effects on vascular structure, endothelial function, or inflammatory mediators,²¹ suggesting that BP lowering did not play a role in our study. The direct beneficial effect of DHA on vascular wall demonstrated in our study is also supported by previous data showing that DHA improved endothelial dysfunction in blood vessels from stroke-prone spontaneously hypertensive rats with only slight BP lowering,²² once more suggesting that BP appears not to play a role in these findings.

In contrast to the antioxidant effect of DHA on vascular wall shown in this and other studies,¹⁹ DHA is highly unsaturated and therefore sensitive to lipid peroxidation.^{23,24} Thus the potentially countervailing actions of the antioxidant effect of DHA on the vasculature and an increase in lipid peroxidation in plasma, which could increase oxidative stress, may be explained by the fact that an important physiological function of DHA in the vascular wall is to protect and maintain membrane phospholipid content during enhanced oxidative stress.^{25–27} The decrease in plasma total cholesterol concentrations in DHA-fed rats may lead to lower free radical production in the wall of blood vessels, which is supported by the findings of Ohara et al.²⁸ These authors reported that free radical generation correlated positively with plasma total cholesterol concentration.

Development of atherosclerosis may be aggravated by BP elevation and over time may contribute to the maintenance of hypertension. The early stages of atherosclerotic disease are associated with increased attraction and adhesion of monocytes to the endothelium by enhanced expression of adhesion molecules such as VCAM-1 and ICAM.²⁹ These adhesion molecules are redox-sensitive and inhibited by nitric oxide.^{5,6,30} Ang II induces their production and secretion through generation of ROS and suppression of nitric oxide.^{5,6,30} In the present study, we demonstrated that DHA inhibits proinflammatory gene expression in Ang II-infused rats, in agreement with results from Staels et al,¹¹ who

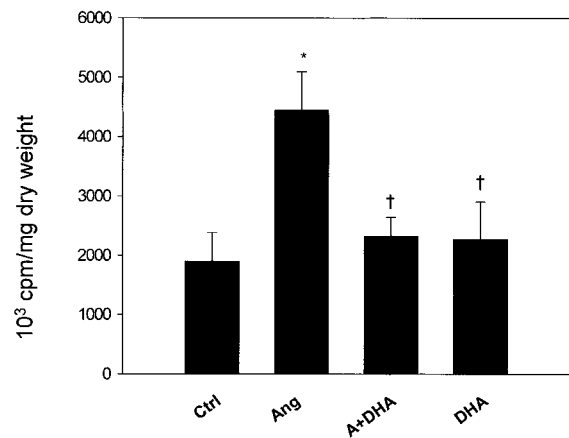


Figure 4. Activity of NADPH oxidase in aortic segments from Ang II-infused rats treated with or without DHA for 7 days. Results are mean \pm SEM (n=5 to 8 per group). * $P < 0.05$ vs control, † $P < 0.05$ vs Ang II group.

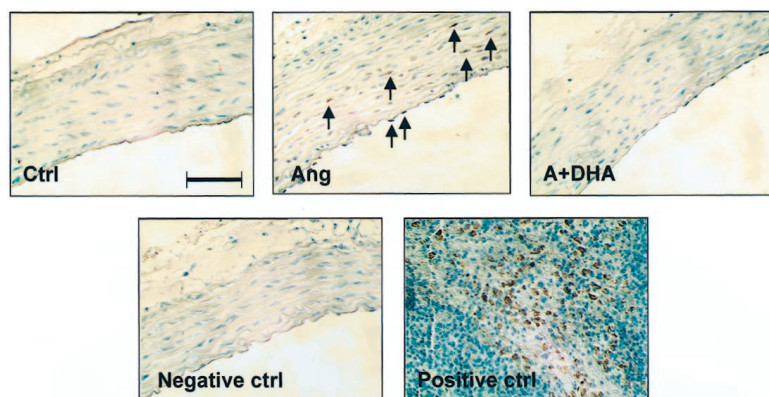


Figure 5. Representative immunohistochemical staining for ICAM in aorta of Ang II-infused rats with or without DHA. DHA alone (similar to A+DHA) is not shown. Positive staining for ICAM appears as brown spots in the Ang II panel as shown by arrows, whereas nuclei appear in all panels as light blue spots. Negative controls were incubated with nonimmune rabbit IgG in place of primary antibody. Spleen was used as positive control for ICAM. Bar=100 μ m.

showed that PPAR α activators inhibit the inflammatory response in the vascular wall partially by repressing NF κ B signaling. PPAR α activators also inhibit cytokine-induced VCAM-1 expression in human endothelial cells,³¹ supporting a role of PPAR α in the prevention of early processes involved in atherosclerotic lesion development.

DHA exerts antihypertensive effects in other hypertensive models.⁸ Alteration of the phospholipid profile in different organs may contribute to the antihypertensive effect of DHA.³² There is a large body of evidence that demonstrates the role of the kidney in regulating blood pressure and the ability of oxidative stress to interfere with tubuloglomerular feedback. Because PPAR α is abundant in the kidney, the antihypertensive effect of DHA could in part be mediated by activation of PPAR α in the kidney. Another possible mechanism underlying BP lowering by DHA could be that it is the precursor of the 3-series prostaglandins, which are platelet antiaggregators and vasodilators. DHA also suppresses Ca²⁺ mobilization in smooth muscle cells.³³ DHA may have a direct relaxant action contributing to BP lowering.^{34,35} DHA had no systemic effects on PRA or plasma aldosterone in Ang II-infused or control rats. In contrast, Engler et al⁸ demonstrated that plasma aldosterone was decreased after DHA feeding to spontaneously hypertensive rats, a discrepancy that could be attributed to use of different models of hypertension.

Engler et al suggested that effects of DHA on steroid and eicosanoid metabolism might be mechanisms whereby this fatty acid prevents hypertension in growing spontaneously hypertensive rats. However, in Ang II-induced hypertension, it is likely that direct effects of DHA on the vascular wall are one of the major mechanisms for its BP-lowering effect.

Perspectives

We have demonstrated that DHA, an ω -3 unsaturated fatty acid and PPAR α activator, has direct beneficial effects on the vascular wall through inhibition of oxidative stress generation and anti-inflammatory action, in addition its involvement in lipid metabolism. This suggests that PPAR α activation may represent an approach to protect the vasculature in hypertension and in early stages of atherosclerosis. Since PPAR α is abundant in the kidney, it remains to be established whether activation of PPAR α in the kidney could contribute to the antihypertensive effect of PPAR α activation. This study opens new avenues for exploration of molecular signaling events in the kidney and elsewhere in Ang II-induced hypertension and from a clinical perspective, for dietetic or pharmacological intervention in the treatment and prevention of blood pressure elevation and progression of atherosclerosis.

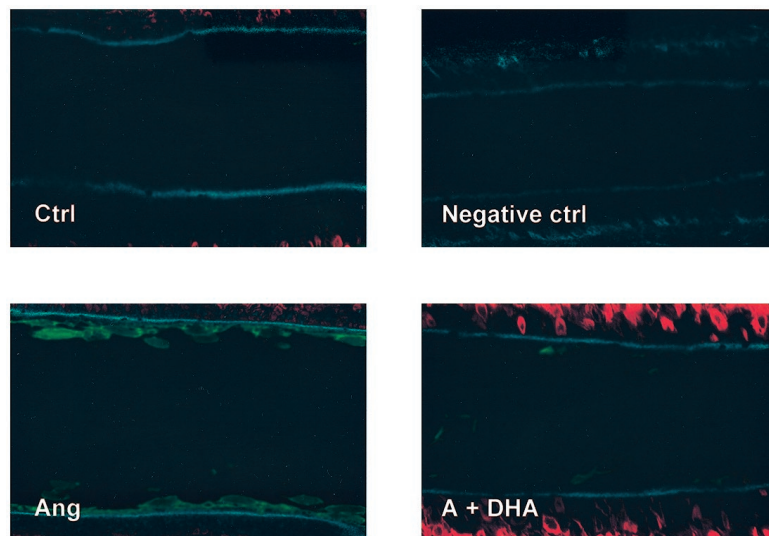


Figure 6. Representative confocal microscopic images for VCAM-1 in mesenteric arteries of Ang II-infused rats with or without DHA. DHA alone (similar to A+DHA) is not shown. Optical section taken longitudinally through the midpoint of an artery wall shows expression of VCAM-1 (green) in endothelium. VSMCs in media are red.

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