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Basic nutritional investigation

Eicosapentaenoic acid prevents salt sensitivity in diabetic rats and decreases oxidative stress

Marianela Vara-Messler [a], Jorge H. Mukdsi [b], Natalia I. Osieki [b,c], Evangelina Benizio [b,c], Gastón M. Repossi [b,c], Ebenezer I.O. Ajayi [d,e], Néstor H. García [b,*]

[a] University of Turin, Turin, Italy
[b] Instituto de Investigaciones en Ciencias de la Salud de Córdoba, Universidad Nacional de Córdoba, Córdoba, Argentina
[c] Instituto de Biología Celular, Cátedra de Biología Celular, Histología y Embriología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina
[d] DC&ONID, Biochemistry Department, Osun State University, Osogbo, Nigeria
[e] IMMF-INIMEC-Universidad Nacional de Córdoba, Consejo Nacional de Investigaciones Científicas y Técnicas, Córdoba, Argentina

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ABSTRACT

Objectives: Salt sensitivity (SS) is associated with increased cardiovascular risk in patients with Type 2 diabetes mellitus (T2-DM) due to an increase in renal oxidation. ω-3 polyunsaturated fatty acids have shown antioxidant effects, but a typical Western diet contains limited content. In particular, ω-3 polyunsaturated fatty acids are able to activate nuclear factor erythroid 2-related factor 2 (Nrf-2) to prevent diabetes mellitus–related complications by mitigating oxidative stress. Therefore, we hypothesized that eicosapentaenoic acid (EPA; ω-3) modulates SS in rats with T2-DM by decreasing renal oxidative stress via Nrf-2 activation and enhancing the antiinflammatory response via interleukin (IL) 6 modulation.
Methods: Three-month-old male rats (n = 40) were fed with a Normal Na-diet (NNaD) and randomly selected into four groups: Healthy Wistar nondiabetic rats (Wi), diabetic controls (eSS), arachidonic acid-treated eSS (AA; ω-6), and EPA-treated eSS (ω-3). After 1 year, rats were placed in metabolic cages for 7 d and fed a NNaD, followed by a 7-d period with a High Na-diet (HNaD). Systolic blood pressure, body weight, serum IL-6 and reactive oxygen species (ROS) levels were determined at the end of each 7-d period. Glycated hemoglobin (HbA1c), triacylglycerol, creatinine, and cholesterol levels were determined. ROS levels and Nrf-2 expression in kidney lysates were also assayed. Histologic changes were evaluated. A *t* test or analysis of variance was used for the statistical analysis.
Results: After a HNaD, systolic blood pressure increased in both the control eSS and AA groups, but not in the EPA and Wi groups. However, HbA1c levels remained unchanged by the treatments, which suggests that the observed beneficial effect was independent of HbA1c levels. The IL-6 levels were higher in the eSS and AA groups, but remained unaltered in EPA and Wi rats after a HNaD diet. Interestingly, EPA protected against serum ROS in rats fed the HNaD, whereas AA did not. In kidney lysates, ROS decreased significantly in the EPA group compared with the eSS group, and Nrf-2 expression was consistently higher compared with the AA and eSS groups. Diabetic rats presented focal segmental sclerosis, adherence to Bowman capsule, and mild-to-moderate interstitial fibrosis. EPA and AA treatment prevented kidney damage.
Conclusions: An adequate ω3-to-ω6 ratio prevents SS in diabetic rats by a mechanism that is independent of glucose metabolism but associated with the prevention of renal oxidative stress generation. These data suggest that EPA antioxidant properties may prevent the development of hypertension or kidney damage.

Introduction

Diabetes mellitus (DM) is a major cause of death and disability worldwide and a strong risk factor for cardiovascular disease. In particular, diabetic nephropathy (DN) remains a significant problem despite efforts to limit its impact on end-organ damage. In a complex milieu where no single treatment can halt DN progression, interactions have been found between metabolic and hemodynamic factors involved in the development of renal lesions in patients with DM [1].

Salt sensitivity (SS), defined as an increase of >10% of blood pressure and secondary to sodium load, is one of the initial changes observed during the development of hypertension in DM.

*Corresponding author. Tel.: +54 [351] 576-0248; fax: +54 [351] 576-0248.
E-mail address: garcia_nestor@hotmail.com (N.H. García).

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According to the classic concept by Guyton and Coleman [2], high salt intake increases a circulating volume, which leads to an increase in renal perfusion pressure, immediately followed by an increase in natriuresis that restores the circulating volume. This pressure-natriuresis mechanism prevents an increase in blood pressure (BP) that could arise from a transient increase of circulating volume. Thus, the deterioration of this mechanism increases the circulating volume and blood pressure leading to hypertension [3].

Several studies have reported the infiltration of macrophages and proinflammatory cells in the kidney at different stages of DN. The inflammatory infiltrate produces reactive oxygen species (ROS) and proinflammatory cytokines, which lead to an upregulation of chronic systemic inflammation and mediate DN progression [4]. As a consequence of the inflammation, various cytokines and acute phase proteins are released to augment or attenuate the inflammatory response. The main inflammatory cytokines involved in the development of DN are interleukin (IL) 6, as well as IL-1β, IL-18, and tumor necrosis factor-α that may contribute to the progression of renal injury, either directly or indirectly [5]. Thus, chronic inflammation of kidney tissue contributes to DN, not only as a consequence of a direct effect of proinflammatory mediators on cellular signaling, but also by creating a state of oxidative stress, and sodium reabsorption is increases under these conditions [6].

In recent years, substantial evidence has implicated nuclear factor erythroid 2-related factor 2 (Nrf2), a redox-sensitive transcription factor, in inflammation and associated disorders. In this setting, the therapeutic potential of Nrf2 activation in DM as relating to the control of oxidative stress has been described [7,8]. Chronic inflammation and oxidative stress contribute not only to DN development, but also to increased sodium reabsorption and enhancing circulatory volume [6], a condition associated with abnormal pressure natriuresis. It is widely believed that abnormal pressure natriuresis is the initial abnormality observed before the fully development of hypertension [9]. In this setting, the relative contribution of interindividual differences on the basis of genetic background, nutrition, physical activity, and other environmental factors has not been fully elucidated.

Understanding how these factors interact is necessary to tackle the global burden of hypertension triggered by DM. In particular, the pathophysiological effects of diets have drawn attention in response to the increasing worldwide adoption of the Western diet and the accompanying increase in the incidence rate of obesity, which is an associated outcome of DM [10]. Particularly, a high intake of ω-6 polyunsaturated fatty acids (PUFAs) and lower intake of ω-3 PUFAs, which is typical of a Western diet, exert several functions that play significant roles in inflammation, metabolism, and the regulation of intracellular processes. The supplementation of eicosapentaenoic acid (EPA 20:5, ω-3) especially is an important regulator of cardiovascular health because of the decrease in the levels of markers and mediators of inflammation, such as cytokines interleukin-1β and tumor necrosis factor α [11]. Therefore, we hypothesized that nutritional supplementation with EPA 20:5 ω-3 prevents the increase of blood pressure owing to sodium load in rats with Type 2 DM (T2-DM) by decreasing renal oxidative stress via Nrf2 activation and decreasing IL-6 release.

Methods

Experimental design

Diabetic rats (eSS) are a stable strain derived from Wistar rats a model of T2-DM that is characterized by fasting hyperglycemia, glucose intolerance, hyperinsulinemia, and early hypertriacylglycerolmia. Diabetic symptoms in this model worsen with age as insulin release decreases and closely resembles T2-DM in adult humans. eSS rats were kindly provided by Professors Tarres and Martinez from the University of Rosario in Argentina [12]. Three-month-old male Wistar (healthy control) or eSS rats (150−200 g) were randomized and housed in cages in groups of four. Animals were maintained under standard environmental conditions (12 h light/dark cycles, at 20−25°C with controlled humidity), and provided the standard isocaloric chow diet and water, both *ad libitum*. Body weight was determined once a week. The study was conducted in accordance with the guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals at the School of Medicine, University of Cordoba in Argentina (Protocol approval: CICUAL 62/18).

Animal treatment

Three-month-old male rats (n = 40) were fed a normal Na-diet (NNaD; 0.4% NaCl; rat/mouse chow diet by GEPSA, Pilar Group, Argentina), and divided into four groups, to be supplemented with different PUFAs by intraperitoneal injection (ip): Wistar rats (Wi) were used as healthy controls, diabetic controls (eSS), eSS treated with arachidonic acid (AA; 20:4 ω-6; (2.5 mg, ip monthly), and eSS treated with EPA (20:5 ω-3; 2.5 mg/ip monthly). After 1 y of treatment, the rats were placed in metabolic cages to acclimatize for 7 d, followed by a first metabolic period feeding with a normal Na-diet (NNaD; 0.4% NaCl), and again another 7 d period with a high Na-diet (HNaD; 4% NaCl), which contained granular sodium chloride (JT Backer, Argentina) that was manually mixed with the regular diet.

Systolic blood pressure (SBP, mmHg), body weight (g), serum IL-6 levels (pg/mL), and ROS serum levels (were determined after the NNaD and HNaD periods. In addition, glycated hemoglobin (HbA1c), triacylglycerol (TAG), creatinine, and cholesterol (Chol) levels (n = 10) were assayed after the NNaD period. After SBP and blood testing was completed, oxidative stress was determined through the assessment of ROS levels in kidney lysates using fluorescence in the presence of dichlorodihydrofluorescein diacetate ($DCFH_2DA$; n = 4 per group). Furthermore, immunohistochemistry for Nrf2 expression was performed in fixed kidney samples (n = 3 per group).

Systolic blood pressure determination

SBP was determined at the end of the NNaD and HNaD periods using the tail-cuff method (NIBP Blood Pressure Systems, Model I229) as previously reported [13]. Briefly, in a quiet room, animals were trained on tail-cuff inflation procedures for 1 wk before the determination of the final blood pressure. Each rat was placed in a plastic restraint maintained at 33°C to 36°C, with its tail passing through the optical sensor and compression cuff and then taped to the platform. The cuff was connected to a blood pressure monitor. On inflation, the cuff stopped the blood flow through the tail, and upon deflation, the sensor detected the reappearance of the blood flow. The results are expressed as mean ± standard error of mean (SEM) from three readings.

Serum lipid, blood glucose, and glycosylated hemoglobin determination

Peripheral blood samples were obtained to determine postprandial glucose, HbA1c, TAG, creatinine, and Chol levels using commercially available kits, as previously reported [13]. After sampling, rats were euthanized and sacrificed by decapitation.

Serum interleukin-6 detection

Before and after the HNaD, blood was drawn from the rat tail vein and contained into vacutainers without anticoagulant. Serum was separated by centrifugation at 3000 × g for 15 min, and maintained at -80°C to determine IL-6 levels (n = 10). IL-6 content was analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, SACCO srl Cadorago, Italy) according to the manufacturer's instructions. Standards with known amounts of IL-6 were used to convert values into absolute concentrations of IL-6 in pg/mL. IL-6 levels are shown as mean ± SEM from three readings [14].

Intracellular reactive oxygen species determination in kidney and serum

Intracellular ROS was also determined in serum and kidney lysates (n = 4) harvested at the end of the protocol. ROS levels were determined with the $DCFH_2DA$ method. Briefly, kidney lysates were homogenized at 4°C in a Tris-buffered saline buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% [v/v] Nonidet *P*-40; 25 mM NaF; 0.5% [w/v] sodium deoxycholate; 10% [w/v] SDS; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM orthovanadate; and 10 mM sodium pyrophosphate) and centrifuged at 1000 × g for 10 min at 4°C.

The supernatant was incubated with 50 μM $DCFH_2DA$ for 30 min at 37°C in the darknes. (MIF) was determined using a PerkinElmer luminescence

spectrometer at an excitation wavelength of 485 nm and emission wavelength of 538 nm. To correct background fluorescence, samples were incubated under the same conditions but without fluorescent dyes. The results were normalized by protein concentration (MIF/mg protein) and expressed relative to the control group. Protein concentrations were quantified with the Bradford assay (Bio Rad reagent) [15].

Nrf-2 expression

Because oxidative stress is a major pathogenic and aggravating factor for kidney diseases, Nrf-2 expression has been proposed as a therapeutic target for renal protection. Nrf-2 expression was evaluated by immunohistochemistry in stained slides (4-μm section) from kidney tissue that was previously fixed and embedded. After antigen-unmasking treatment, the sections were washed and incubated with the hamster antirat Nrf-2 (1:100) monoclonal antibody.

After washing with a Tris-buffered saline buffer, the sections were placed on a chromogen diaminobenzidine reaction, giving a brown positive precipitate. Nrf-2-positive cells were counted from the slides of three samples for each condition per 10 microscopic fields (20 × magnification) using an Olympus BH2 light microscope in a blinded manner. Nrf-2-positive cells were expressed as mean ± SEM of Nrf-2-positive cells per field [16].

Histologic study

After removal, the right kidney was fixed by a 4% paraformaldehyde infusion. The tissue was embedded in paraffin for assessment with light microscopy and immunohistochemistry. Sections of 2-3 μm were cut and stained with hematoxylin-eosin and periodic acid-Schiff. Glomerular damage (i.e., the presence of fibrosis, adherence to the capsule, and mesangial expansion and proliferation), interstitial mononuclear cell infiltration, and arteriolar thickening were assessed. A minimum of 100 glomeruli were evaluated in each kidney.

The pathologist was blinded to the sample and used × 40 resolution to grade severity on a scale from 0 to 3 as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. An average score was obtained for both glomerular and interstitial changes.

Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments. The statistical analysis was performed using a *t* test (two-tailed probability value) to compare two groups, or analysis of variance for multiple groups using GraphPad Prism version 8.00 for Windows (San Diego, CA. A *P*-value ≤ 0.05 was considered statistically significant.

Results

HNaD increased and EPA prevented blood systolic pressure in diabetic rats

Wi rats weighed 37% more than eSS rats of the same age; however, no difference was observed in basal SBP. As expected, rats with T2-DM showed higher HbA1c levels (+43%) compared with those in the Wi group. Additionally, the eSS group showed higher postprandial glucose, Chol, and TAG levels. All rats with T2-DM that were treated had lower serum lipid levels compared with those in the eSS group. Renal functions were similar between the groups. Table 1 shows the physiological parameters of each group before HNaD feeding.

Next, we evaluated the effect of sodium load on SBP (Fig. 1). HNaD increased SBP in the eSS group from 109 ± 6 mm Hg to 121 ± 5 mm Hg (11.2% ± 2.3%; $P \leq 0.01$) as well as in the AA-treated group (from 120.0 ± 4.0 mm Hg to 133.0 ± 2.0 mm Hg; 10.2% ± 1.6%, $P \leq 0.003$). By contrast, SBP did not change in the EPA-treated (from 126.0 ± 2.0 mm Hg to 129.0 ± 2.0 mm Hg; 2.9% ± 3.8%; $P > 0.28$) and Wi (from 115.0 ± 6.0 to 117.0 ± 5.0 mm Hg, 1.7% ± 2.1%; $P > 0.8$) groups. No difference in food intake was observed between the groups (average intake: 18 ± 1 gr/d/rat) during the metabolic studies. Thus, EPA treatment prevented increase in SBP in rats with T2-DM after sodium load. With these data, we obtained insight into the mechanism by which EPA prevents increase in SBP.

Table 1
Physical characteristics and biochemistry parameters of animal groups after NNaD

	Wistar (n = 10)	eSS (n = 10)	eSS + AA ω-6 (n = 10)	eSS + EPA ω-3 (n = 10)
Weight (g)	551 ± 4	402 ± 22	425 ± 26	506 ± 20
SBP (mmHg)	115 ± 6	109 ± 6	120 ± 4	126 ± 2
HbA1c (%)	4.5 ± 0.1	6.43 ± 0.2*	6.2 ± 0.2*	6.1 ± 0.2*
Postprandial glucose (mg/dL)	118 ± 1.7	136 ± 4.0*	126 ± 3.6*	113 ± 2.7*,‡
Serum TAG (mg/dL)	113 ± 1.5	192 ± 11.2*	160 ± 25.0*,†	141 ± 4.9*,†
Serum Chol (mg/dL)	41.2 ± 26.7	92.3 ± 2.8*	61 ± 3.5*,†	68.3 ± 8.6*,†
Serum creatinine (mg/dL)	0.68 ± 0.07	0.55 ± 0.02	0.62 ± 0.07	0.60 ± 0.05

AA, arachidonic acid; Chol, cholesterol; EPA, eicosapentaenoic acid; eSS, diabetic control; HbA1c, glycosylate hemoglobin; HNaD, high Na-diet; NNaD; normal Na-diet; SBP, systolic blood pressure; SEM, standard error of mean; TAG; triacylglycerol; Wi, Wistar rats
Three-month-old eSS rats (n = 40) were fed with a NNaD (0.4% NaCl) and divided into three groups: eSS, eSS treated AA (20:4 ω-6), eSS treated with EPA (20:5 ω-3), and Wi (healthy controls; n = 10). After 1-year treatment with fatty acids, rats were placed for 7 d in metabolic cages and fed an HNaD (4% NaCl). Before that, body weight, SBP, posprandial glucose (mg/dL), as well as HbA1c, TAG, and Chol levels were determined. Values are mean ± SEM (n = 10 per group).
*$P \leq 0.05$ versus Wistar
†$P \leq 0.05$ versus eSS
‡$P \leq 0.05$ versus eSS + ω-6

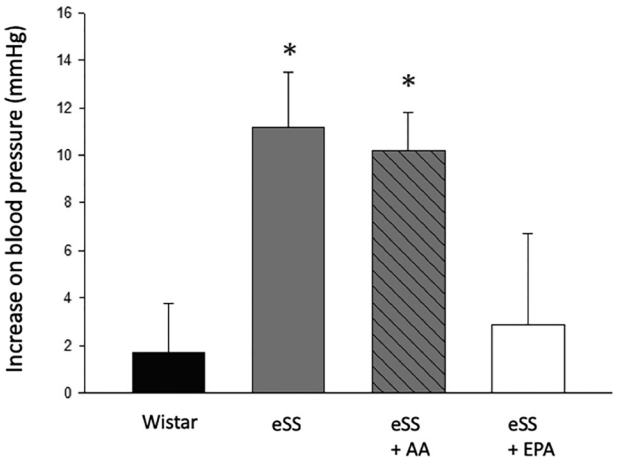

Fig. 1. Effect of high sodium diet on blood pressure. Rats were underwent to a high Na-diet for 1 week. SBP increased only in diabetic control (eSS) and arachidonic acid-treated rats (eSS+AA). Eicosapentaenoic acid prevented increasing SBP. *$p \leq 0.05$ versus eSS+ EPA (n = 10) and Wistar rats (n = 10).

EPA decreased serum interleukin-6 and ROS

Because slowchronic inflammation may increase blood pressure, we investigated whether this elevation of SBP was associated with an inflammatory mechanism. Therefore we determined serum IL-6 levels after the NNaD and HNaD periods. HNaD increased IL-6 levels in eSS treated rats from 2.0 ± 0.8 to 2.4 ± 0.1 pg/mL and in AA from 1.6 ± 0.1 to 2.0 ± 0.1 pg/mL. In EPA-treated rats, as well as in healthy Wi rats, IL-6 levels were not increased after HNaD (1.3 ± 0.1 versus 1.4 ± 0.1 pg/mL and 1.4 ± 0.1 versus 1.3 ± 0.1 pg/mL, respectively) (Fig. 2).

To add further evidence that slow chronic inflammation mechanism is involved on the increase of SBP, we determined serum ROS levels (Fig. 3 a) after the NNaD and HNaD periods. Serum ROS levels in eSS rats increased with HNaD, as well as in AA-treated eSS rats. By contrast, EPA-treated animals did not show higher ROS levels after HNaD. These results indicate that EPA prevents the increase of serum IL-6 and ROS in eSS rats after sodium load.

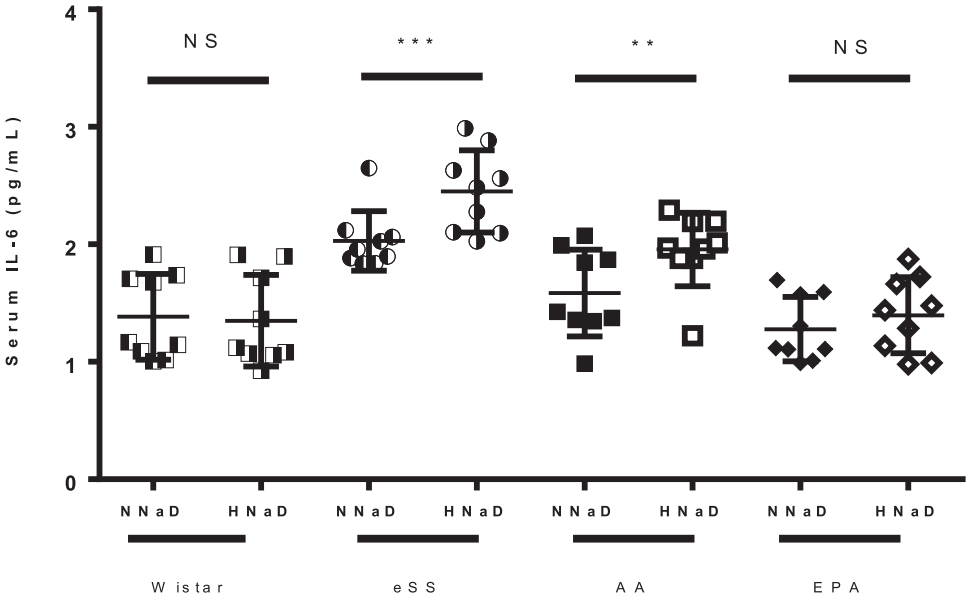

Fig. 2. Serum interleukin (IL)-6 determination before and after HNaD. Blood samples were drawn from the vein tail and separated from serum by centrifugation. Subsequently, serum was maintained to determine IL-6 levels by ELISA. Dots show IL-6 pg/mL expressed as mean ± SEM; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ normal versus HNaD (*t* test; n = 10).

EPA decreased kidney reactive oxygen species

The kidney is a major effector of the pressure natriuretic mechanism and serum changes may not represent changes on the interstitial kidney. Therefore, we investigated the effect of EPA treatment on ROS levels in kidney tissues. At the end of the HNaD period, ROS levels were determined in kidney lysates (Fig. 3b). The EPA-treated group showed a lower (-84.8%; $p \leq 0.05$) ROS level with respect to eSS, which suggests that during the HNaD, intrarenal ROS production is limited compared with eSS. Although the AA-treated group showed decreased ROS levels compared with the eSS group, ROS levels were still higher with respect to those of EPA-treated rats.

EPA enhanced Nrf-2 expression

Nrf2 is a protein that can regulate cell antioxidant response against oxidative damage, is triggered by injury and inflammation, and can be stimulated by several drugs. We evaluated the effect of EPA supplementation on Nrf2 kidney expression after animals were loaded with a HNaD. Figure 4 shows Nrf2 expression in the kidney, and Table 2 and Figure 5 show changes in the kidneys from sodium-overloaded rats with T2-DM after treatment with fatty acids. In rats supplemented with EPA, Nrf-2 expression was higher compared with eSS rats (124 ± 8.6 vs. 14 ± 1 positive cells/field) and AA treatment (4.9 ± 0.39 positive cells/field). Nrf2 expression in Wi rats was similar to that with EPA.

Histologic evaluation

To evaluate whether these effects were associated with morphologic changes, kidneys from all groups were evaluated (Fig. 5; Table 2). As expected, the kidneys of untreated diabetic rats showed significant glomerular changes with focal segmental sclerosis and adherence to Bowman capsule. At the interstitial level, the kidneys exhibited mild-to-moderate interstitial fibrosis.

However, after EPA and AA treatments, a significant improvement was observed, not only on the glomerular but also the interstitial damage. At the glomerular level, the mesangial matrix was expanded in segmental localization, and interstitial fibrosis was either absent or focal and mild. These findings suggest that EPA supplementation has an antiinflammatory effect and is associated with morphologic changes.

Discussion

Our results show that EPA treatment minimized inflammation and oxidative stress in rats with T2-DM through Nrf2 activation during sodium load, which are effects associated with less glomerular sclerosis and less interstitial fibrosis. This effectively prevented SS observed in untreated diabetic rats and was independent of glucose homeostasis because HbA1c levels did not change. Particularly, EPA supplementation prevented the deleterious outcome by improving endothelial function and preventing increased blood pressure in rats with DM after sodium load.

HNaD increased SBP in eSS rats while EPA supplementation prevented increase in blood pressure. By contrast, AA did not. According to the American Heart Association, SS occurs when blood pressure increases in response to changes in salt intake and it is “a risk factor for cardiovascular mortality and morbidity, independentely of the BP” [17].

Although the identification criteria are not standardized, SS is defined as a change in blood pressure (office measurement) of 10% or at least 5 mm Hg in response to a change in NaCl [18]. Another definition of SS is an increase in mean arterial blood pressure (MAP) of at least 4 mm Hg (24-h ambulatory blood pressure monitoring) with an increase in NaCl intake [19]. An SS index (i.e., difference between MAP on low- and high-salt diets, divided by MAP on a low-salt diet) of at least 5% is also another definition of SS [20]. However, the most reliable method to measure SS is blood pressure response before and after a change in dietary salt intake [21].

Hypertension and SS are both conditions that result from a genetic predisposition combined with environmental influences, such as excessive sodium consumption and sedentary lifestyles. Approximately one-third of the world’s population has hypertension, which causes almost 50% of deaths from stroke and coronary

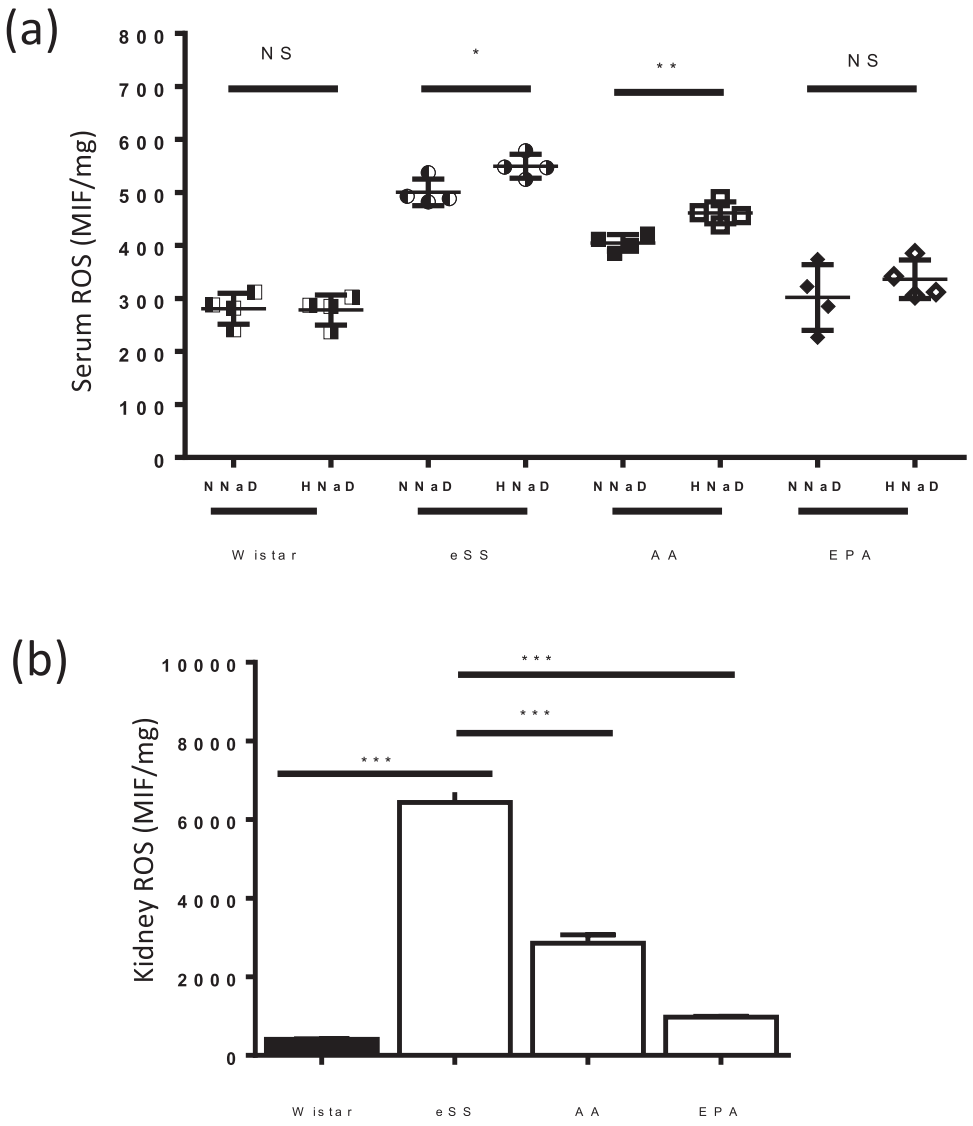

Fig. 3. Intracellular ROS production in T2-DM-bearing rats. ROS levels in (a) Serum from T2-DM-bearing rats before and after a sodium overload. Dots show MIF/mg expressed as mean ± SEM, $^{*}p \leq 0.06$ and $^{**}p \leq 0.01$ NNaD versus HNaD. t-Test. n = 4. (b) Kidney tissues lysates after treatment with fatty acids and sodium overload. Bars show MIF/mg expressed as mean ± SEM, $^{***}p \leq 0.001$ eSS versus Wistar, AA or EPA. ANOVA. n = 4.

heart disease [22]. However, these statistics do not distinguish SS from salt-resistant hypertension or include normotensive patients with SS. This distinction is important because SS, independent of blood pressure, is a risk factor for cardiovascular morbidity and mortality [23,24] and other diseases (e.g., asthma, gastric carcinoma, osteoporosis, and renal dysfunction) [25]. Therefore, strategies to prevent the development of SS may be cost effective.

Almost 5 decades ago, Guyton and Coleman [2] proposed that SS is the result of kidney malfunction. However, recent studies suggest that nonosmotic salt accumulation in the skin interstitium [3] and changes in endothelial surface layer characteristics, which lead to an alteration of the endothelial cell function [26,27], also playing an important role in the nonosmotic storage of salt. Other investigators [28,29] have identified two novel pathways in SS hypertension: β2-adrenergic stimulant-glucocorticoid receptor-with-no-lysine kinase 4-Na+-Cl- cotransporter pathway and renin-angiotensin system-related C3 botulinum toxin substrate 1-mineralocorticoid receptor pathway, which are both active in distal convoluted tubule, connecting tubules, and collecting ducts. These new concepts emphasize that sodium homeostasis and SS appear related not only by kidney malfunction but also by endothelial dysfunction.

A chronic high-sodium diet induces kidney damage at least via the formation of ROS, such as superoxide, hydrogen peroxide, and peroxynitrite, which increase sodium reabsorption by reducing nitric oxide (NO) bioavailability [30] or enhancing transport in an NO-independent manner via protein kinase C alpha activation [31,32]. Either mechanisms increase electrolyte retention and extracellular fluid volume [6]. Additionally, a long-term HNaD increases ROS derivatives and can increase urinary protein excretion and decrease endothelial response to acetylcholine [13] in the absence of hypertension, which demonstrates that HNaD by itself induces tissue damage.

Our results demonstrate that an acute load of sodium in a diabetic setting has the unfavorable effect of at least increasing tubular oxidative stress and consequently SS. Although this increase was not observed in animals treated with EPA (ω-3), AA (ω-6) supplementation did not prevent the increase of ROS or SS. Hypertension is twice as frequent in patients with diabetes compared with

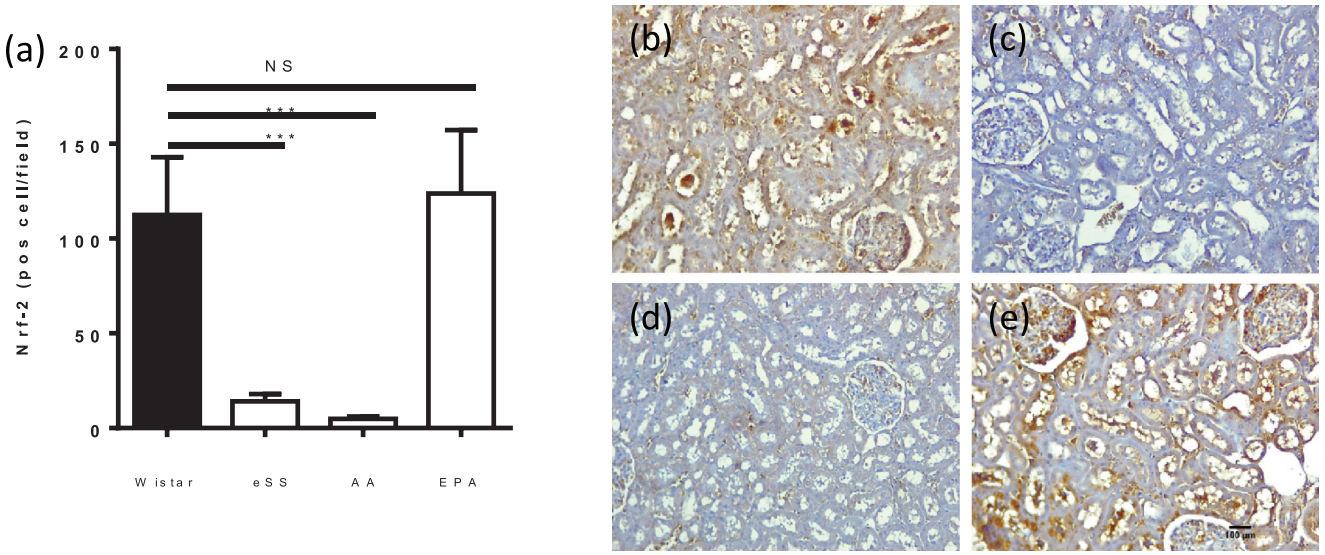

Fig. 4. Nuclear factor erythroid 2-related factor 2 (Nrf2) expression in kidney tissues from sodium-overloaded rats with type 2 diabetes mellitus after treatment with fatty acids. (a) Immunolabeling for Nrf2 expression in kidney tissues from rats with Type 2 diabetes mellitus. Bars show Nrf2+ cells/field expressed as mean ± standard error of mean; $^{***}p \leq 0.001$ Wistar versus diabetic control, arachidonic acid, or eicosapentaenoic acid (analysis of variance; n = 3). Representative images (20 × magnification) from (b) Wistar, (c) diabetic control, (d) arachidonic acid, and (e) eicosapentaenoic acid. Tissues were collected from rats and fixed in paraformaldehyde 4% for immunostaining. Nrf2+ cells were counted from tissues.

Table 2
Morphologic changes in kidneys from rats with T2-DM after treatment with fatty acids and a sodium overload

	Wistar (n = 10)	eSS (n = 10)	eSS + AA ω-6 (n = 10)	eSS + EPA ω-3 (n = 10)
Glomerular sclerosis (0–3)	0 ± 0	0.75 ± 0.64*	0.01 ± 0.01†	0 ± 0†
Renal interstitial infiltration (0–3)	0 ± 0	1.33 ± 0.33*	0.67 ± 0.21	0.25 ± 0.25†

AA, arachidonic acid; EPA, eicosapentaenoic acid; eSS, diabetic control
Values are mean ± standard error of mean
*$P \leq 0.05$ versus Wistar
†$P \leq 0.05$ versus eSS

those who do not have diabetes. The major cause of morbidity and mortality in diabetes is cardiovascular disease, which is exacerbated by hypertension [33]. Even if the role of ω-3 fatty acids in hypertension has been widely studied, data on the role of SS remains scant.

With respect to blood pressure, several observational studies have demonstrated an association between ω-3 fatty acid intake and low blood pressure levels, and interventional studies using ω-3 fatty acid supplementation have shown blood pressure-lowering effects. Of note, a population-based international study of macro- and micronutrients and blood pressure, which surveyed 4680 men and women ages 40 to 59 y from 17 population samples, found an inverse association between blood pressure and ω-3 fatty acid intake [34].

A prospective and interventional study also found that the long-term use of EPA is effective to prevent major coronary events in hypercholesterolaemic patients in Japan who consume a large amount of fish [35]. Other studies have reported both similar and opposite results. A recent meta-analysis by Popoff et al. [36] to evaluate the effect of ω-3 fatty acids on mortality, morbidity and adverse events in patients with acute myocardial infarction found no benefit of ω 3 fatty-acids supplementation. The cause of this contradictory effect may be endothelial health, which suggests that patients with advanced endothelial disease may not respond properly.

A large body of evidence from experimental, clinical, and epidemiologic research has also demonstrated the potential benefits of ω-3 fatty acids, such as EPA on cardiovascular health (including antiatherogenic, antiarrhythmic, and plaque stability effects) as well as the improvement of endothelial and platelet function. In addition, ω-3 fatty acids are able to switch AA-derived eicosanoid profiles and convert ω-3 fatty acids to vasodilators and platelet anticoagulation factors [37].

Many underlying molecular mechanisms that cause micro- and macrovascular complications of diabetes include oxidative stress and inflammation. One of the potential immune mediators in hypertension is IL-6. Activation of the intrarenal renin-angiotensin system has been proposed to facilitate the development of angiotensin II-dependent hypertension, whereas IL-6 contributes to the increase of angiotensinogen expression in cultured renal proximal tubular cells [38]. Moreover, Zhang et al. [39] have demonstrated that intrarenal IL-6 is associated with angiotensin II-dependent hypertension. We found that one involved mechanism could be the modulation of proinflammatory IL-6, where levels were higher in eSS- and AA-treated rats, but not in EPA-treated and Wi rats, after HNaD feeding. Omega-3 fatty acids, such as EPA, have been shown to have beneficial impacts on multiple risk factors linked to T2-DM, including blood pressure and blood vessel functions as well as blood lipid levels due to their antithrombotic, anti-inflammatory and antioxidative actions [40].

The antiinflammatory effects of EPA are mediated through the reduction of AA-derived inflammatory mediators, the activation of nuclear receptor peroxisome proliferator-activated receptor γ, the G-protein-coupled receptor 120 as an agonist [41], and the stimulation of the adenosine monophosphate-activated protein kinase/silence information regulator pathway [42]. In our model, although we did not determine any EPA derivative, a general agreement exists that EPA exhibits antiinflammatory properties through its metabolites in each organ and that EPA has pleiotropic and antiinflammatory effects on various tissues and lesions, such as atherosclerotic lesions [43].

Proinflammatory cytokines, such as IL-6, plays such an important role in hypertension, and oxidant generation is an inherent

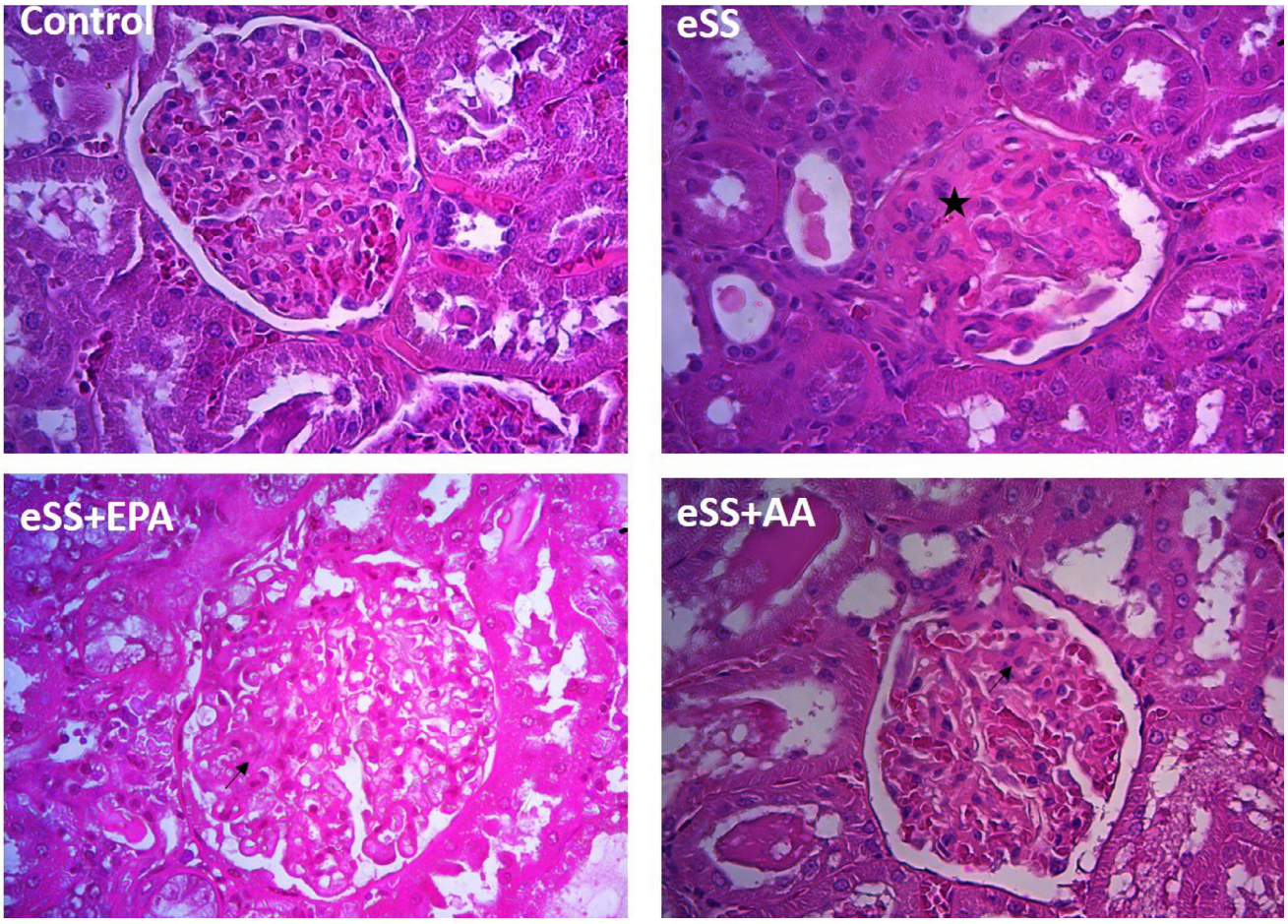

Fig. 5. Representative images of kidneys. Diabetic control animals exhibited significant focal segmental sclerosis (asteric) in relation to control rats. Eicosapentaenoic and arachidonic acid treatments induced an important remission of glomerular lesions, showing only mild and segmental mesangial expansion (arrows; hematoxilin & eosin staining; original magnification 40 ×).

participant in the process of increased sodium reabsorption and increased blood pressure [6]. Consistently, we also demonstrated that EPA treatment can protect against increases in serum ROS levels as shown by many other investigators [44–46].

In eSS rats, ROS levels increased when feeding the HNaD, but decreased significantly in EPA-treated rats compared with the eSS and AA-treated groups. Oxidative stress-induced complications of diabetes have been demonstrated to include stroke, neuropathy, retinopathy, and nephropathy. Elevated ROS levels in diabetes may be due to an increase in the production and/or decrease in the destruction by catalase, superoxide dismutase, and glutathione peroxidase antioxidants [47]. In particular, oxidative stress is believed to play an important role in the development of vascular complications in T2-DM. Alterations in redox homeostasis through an increased intake of ω-3 fatty acids have been linked to the activation of the Nrf2 pathway, by which this transcription factor (which is key in regulating glucose and lipid metabolism as well as redox homeostasis) induces the transcription of endogenous antioxidants [48].

We observed Nrf2 expressions in the kidney. In our model, Nrf2 expression was higher in EPA-treated rats compared with the AA-treated and eSS groups, which shows that Nrf2 is involved in the modulation of ROS. In line with our finding, the comparative metabolic effects of a diet rich in saturated fat versus an ω-3-enriched diet in a mice model that overexpresses the endogenous antioxidant catalase was recently reported. The 8-wk dietary intervention showed that mice overexpressed endogenous catalase when fed an ω-3 fatty acid–enriched diet, as opposed to a saturated fat diet, and activated G-protein-coupled receptor 120-Nrf2 crosstalk to maintain a balanced energy metabolism, normal circadian rhythm, and insulin sensitivity, and thereby reduce the risk of metabolic syndrome and associated diseases compared with their wild-type controls [49]. Furthermore, Nrf2 is a redox-sensitive transcription factor activated by long-chain fatty acids (including EPA), phenolic antioxidants, and imbalances in redox stress. Increasing levels of Nrf2 by endogenous production of electrophilic products or pharmacologic agents have been shown to prevent or act as therapies for T2-DM and cardiovascular disease through activating antiinflammatory pathways [50].

Low-grade inflammation is a common feature of kidney disease, which is typically already present before the need to start renal replacement therapy, and evidence suggests that persistent inflammation may also be, *per se*, a risk factor for the progression of chronic kidney disease (CKD) and vascular disease. Many factors, including the retention of proinflammatory cytokines, advanced glycation end products, reactive oxygen species, autonomic dysfunctions, and volume overload, may contribute to inflammation when renal function declines. As noted, Nrf2 plays a central part in basal activity and the coordinated induction of several genes that encode antioxidant and phase 2 detoxifying enzymes, as well as related proteins. Consequently, constitutive Nrf2 activity is critical to maintain redox balance in normal conditions, and its induction in response to oxidative stress is essential to protect against oxidative stress.

However, studies conducted in animals with 5/6 nephrectomy-induced CKD have revealed that despite the presence of oxidative stress and inflammation, which should have induced Nrf2 activation and upregulation of its target genes, animals with CKD exhibited a marked and time-dependent decline in nuclear Nrf2 content, which points to impaired activation in the remaining kidney [51]. This impaired activation of Nrf2 and the expression of antioxidant enzymes can be restored by Olmesartan, an angiotensin II receptor blocker [52]. Under these conditions, Olmesartan therapy prevented nephropathy, suppressed oxidative stress and inflammation, and restored Nrf2 activation and expression of antioxidant enzymes.

Equally, weak Nrf2 activators that are commonly found in foods or dietary supplements have renoprotective effects in rodent models. For example, sulforaphane (organosulfur compound found in cruciferous vegetables) has been shown to improve nephropathy in animals with streptozotocin-induced diabetes [53], mice with antiglomerular basement membrane glomerulonephritis [54], and cisplatin-induced nephrotoxicity [55].

Taken together, our data support the role of Nrf2 deficiency in the pathogenesis of oxidative stress, inflammation, and progression of CKD and the renoprotective effect of Nrf2 activators. New therapeutic approaches that target oxidative stress and inflammation are currently being developed to treat diabetes-associated cardiovascular complications, such as trials using bardoxolone methyl in which a reduction of serum creatinine in patients with kidney damage was observed [56]. However, larger trials are needed to investigate the efficiency of this molecules. These studies are in agreement with our results, and show that mechanisms that activate Nrf2 expression or activity prevent the low-grade inflammation process that may finally develop kidney damage.

Conclusions

Overall, this study provides compelling evidence that adequate ω-3 supplementation can minimize inflammation as well as oxidative stress through Nrf2 activation in T2-DM after Na overload. EPA effectively prevented SS observed in untreated diabetic rats independent of glucose homeostasis because the HbA1c levels did not change. In particular, EPA supplementation did not present any deleterious effect because EPA improved endothelial function and thus prevented increased blood pressure in DM after Na load.

To date, several policies have been proposed to reduce the salt intake of populations to the level recommended by the World Health Organization or other organizations. Some policies have been executed at the population-based level, including reformulation of the foods, taxation, and food labeling, but others have been developed at the individual level, including dietary counseling [57,58] to prevent blood pressure increase and provide better control of the hypertensive population. Because of low patient compliance to low-sodium diets, we evaluated whether supplementation with ω-3 (in particular, EPA) that may help to delay the increase in blood pressure. Overall, supplementation with EPA in normotensive diabetic rats that were fed a high-sodium diet prevented SS via the upregulation of Nrf2 expression.

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