Inducible NOS inhibition, eicosapentaenoic acid supplementation, and angiotensin II–induced renal damage

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Background. Cytochrome P450(CYP)-dependent hydroxylation and epoxygenation metabolites of arachidonic acid (AA) influence renal vascular tone, salt excretion, and inflammation. Transgenic rats over expressing both human renin and angiotensinogen genes (dTGR) feature angiotensin II (Ang II)–induced organ damage, increased expression of inducible nitric oxide synthase (iNOS), decreased AA hydroxylation, and epoxygenation. As nitric oxide production via iNOS can inhibit CYP AA metabolism, we tested the hypothesis that by blocking iNOS or by supplementing eicosapentaenoic acid (EPA), which can serve as an alternative CYP substrate, Ang II–induced vasculopathy could be ameliorated.

Methods. We treated dTGR with the iNOS inhibitor L-N(6)-(1-iminoethyl) lysine (L-NIL), EPA, and the combination of both treatments from week 4 to 7.

Results. Immunohistochemistry showed that L-NIL and EPA reduced glomerular iNOS toward control levels. L-NIL–treated dTGR showed cardiac hypertrophy and albuminuria similar to untreated dTGR. EPA and the combination of EPA + L-NIL, ameliorated organ damage without lowering blood pressure. EPA and EPA + L-NIL reduced cardiac hypertrophy, albuminuria, renal fibronectin expression, and infiltration of monocytes/macrophages, compared to L-NIL and untreated dTGR. Reactive oxygen species were detected in glomeruli of untreated and L-NIL–treated dTGR, but was reduced in the EPA groups. EPA treatment reduced activator protein-1 (AP-1) activation and partially inhibited nuclear factor-kappaB (NF-κB) activity in kidneys of dTGR.

Conclusion. These results demonstrate that iNOS inhibition does not protect against Ang II–induced end-organ damage, while EPA treatment does. Our electromobility shift assay experiments revealed that EPA protection may involve inhibition of AP-1– and NF-κB–dependent pathways.

Cytochrome P450 (CYP)-dependent arachidonic acid (AA) hydroxylation and epoxygenation produces numerous compounds relevant to cardiovascular disease. The metabolites include 19- and 20-hydroxyeicosatetraenoic acid (19- and 20-HETE) and four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12- and 14,15-EET). Members of the CYP4A subfamily produce 20-HETE, whereas the epoxygenation reaction is catalyzed by CYP2C and CYP2J enzymes [1]. 20-HETE and EETs play important roles in the regulation of renal vascular tone, salt excretion, and anti-inflammatory mechanisms [2]. Both 20-HETE and EETs inhibit sodium reabsorption [3, 4]. A deficiency in tubular expression of 20-HETE–generating CYP4A enzymes and failure to up-regulate EET-generating CYP2C enzymes was related to salt-sensitive hypertension in Dahl rats [5, 6]. 20-HETE is also an endogenous vasoconstrictor by inhibiting Ca2+–activated potassium (BK) channels and by activating Rho-kinase [7, 8]. 20-HETE inhibition reduces blood pressure in spontaneously hypertensive rats and deoxycorticosterone acetate (DOCA) salt-treated rats [9, 10]. In contrast, EETs are potent vasodilators. They activate BK channels in vascular smooth muscle cells (VSMCs) and may function as endothelium-derived hyperpolarizing factors (EDHF) in different vascular beds [7, 11]. Moreover, 11,12-EET revealed anti-inflammatory properties by inhibiting tumor necrosis factor-α (TNF-α)–induced nuclear factor-kappaB (NF-κB) activation in coronary endothelial cells [12].

Eicosapentaenoic acid (EPA), a major n-3 polyunsaturated fatty acid (PUFA) in fish oil, may serve as an alternative substrate for the AA-metabolizing CYP enzymes [13]. In analogy to AA metabolism, CYP-dependent EPA conversion results in a set of hydroxylation and epoxygenation products: 19- and 20-OH-EPA and five...
regioisomeric epoxyeicosatetraenoic acids (EETeTrs). The biologic activities of these metabolites have only been partially investigated. Recent studies demonstrated that EPA epoxides share and even exceed the capacity of AA epoxides to activate BK channels, to mediate vasodilation, and to stimulate cardiac adenosine triphosphate (ATP)-sensitive potassium channels [14–16].

We showed earlier that angiotensin II (Ang II) down-regulates CYP-dependent renal AA metabolism [17, 18]. We speculated that AA epoxygenation products with anti-inflammatory properties might thereby be reduced, contributing to uncontrolled inflammation, particularly in the kidney. We relied on a double transgenic rat (dTGR) model expressing both the human renin and angiotensinogen genes; the animals develop severe vasculopathy and die at age 8 weeks [18, 19]. We reasoned that by supplementing EPA, we could ameliorate the down-regulation of CYP-dependent metabolites. Since nitric oxide overproduction by nitric oxide synthase (iNOS) in inflamed tissues inhibits CYP metabolism, we hypothesized that iNOS inhibition would increase the effect of EPA. Therefore, we gave dTGR EPA supplementation, the iNOS inhibitor L-N(6)-(1-iminoethyl)lysine (L-NIL), or both.

METHODS

Experiments were conducted in male dTGR and age-matched Sprague-Dawley rats. The dTGR line and characteristics are described elsewhere [18, 19]. The rats were purchased from RCC (Füllinsdorf, Switzerland), kept in rooms at 24 ± 2°C, were fed with a standard rat diet containing 0.2% sodium by weight, and were allowed free access to tap water. All American Physiological Society guidelines were followed and local authorities (permit # G 408/97) approved the studies. The treatments were started at week 4 and continued for 3 weeks. Twelve vehicle-treàted (0.9% saline) dTGR, 15 L-NIL–treated dTGR (3 mg/kg/day intraperitoneally) according to the dosage used by McCartney-Francis et al [20], 15 EPA-treated dTGR (7.5 g/kg dry rat food), 15 EPA + L-NIL–treated dTGR, and six nontransgenic Sprague-Dawley rats were used in the study. Due to the small size of the rat tail, the first blood pressure measurements were performed at week 5 by tail-cuff under light ether anesthesia 20 hours after the last L-NIL dose. Urine samples were collected over a 24-hour period. Urinary rat albumin was measured by enzyme-linked immunosorbent assay (ELISA) (Celltrend, Lückenwalde, Germany). Rats were killed at age 7 weeks. The kidneys and hearts were washed with ice-cold saline, blotted dry, and weighed. For electrophoretic mobility shift assay (EMSA) of NF-kB and activator protein-1 (AP-1), the tissues were snap-frozen in liquid nitrogen, for immunohistochemistry in isopentane (∼35°C), and stored at −80°C.

Renal microsomal AA and EPA metabolism

Microsomes were prepared from freshly dissected kidneys. AA as well as EPA hydroxylase and epoxygenase activities were determined as described previously [17]. Briefly renal microsomes (80 μg of protein in a total volume of 0.1 mL) were incubated in 50 mmol/L Tris/HCL buffer, pH 7.5, with 10 nmol [1-14C] AA (0.55 × 10^6 dpm) and 10 nmol [1-14C] EPA (0.55 × 10^6 dpm), respectively, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (0.5 mmol/L) for 20 minutes at 37°C. The reaction products were extracted into ethyl acetate and resolved by reverse-phase high-pressure liquid chromatography (RP-HPLC) as described [17]. AA and EPA hydroxylase activities were determined as the sum of 19- and 20-HETE as well as 19- and 20-OH-EPA, respectively. AA and EPA epoxygenase activities were determined as the sum of EETs and corresponding dihydroxyeicosatrienoic acids (DHETs) as well as the sum of the synthesized EETeTrs and corresponding dihydroxyeicosatetraenoic acids (DHETeTr), respectively. All activities are given in nanomoles of the respective metabolites produced per minute and milligrams of microsomal protein.

Immunohistochemistry

Frozen kidneys were cryosectioned at 6 μm thickness and stained by immunofluorescence or the alkaline phosphatase-antialkaline phosphatase (APAAP) technique as described earlier [18, 21]. The sections were fixed with cold acetone, washed with Tris-buffered saline (TBS), and blocked with 10% normal donkey serum (Dianova, Hamburg, Germany) for 30 minutes and incubated with primary monoclonal antibody against monocyte/macrophages (ED-1) (Serotec, Wiesbaden, Germany) for 60 minutes in a humid chamber at room temperature. After washing with TBS, the sections were incubated with a bridging antibody (rabbit-antimouse IgG) (Dako, Hamburg, Germany) for 30 minutes at room temperature and washed again with TBS. The APAAP complex (Dako) was applied and the sections were incubated for 30 minutes at room temperature. The immunoreactivity was visualized by development in a mixture of naphthol-AS-BI-phosphate (Sigma, Schnelldorf, Germany) with neufuchsin (Merck, Darmstadt, Germany). Endogenous alkaline phosphatase was blocked by addition of 10 mmol/L levamisole (Sigma, Germany) to the substrate solution. The sections were slightly counterstained in Mayer’s hemalaun (Merck), blued in a tap water, and mounted with Geffol (Coulterimmunotech, Marseille, France). Semiquantitative scoring of infiltrated ED-1–positive cells in the kidney was performed using computerized cell count program (KS 300 3.0) (Zeiss, Göttingen, Germany). Fifteen different cortical areas of each kidney (N = 5 in all
groups) were analyzed. For quantification of perivascular macrophage infiltration, all selected view fields included a small vessel in their analysis. The samples were examined without knowledge of the rats’ identity.

For immunofluorescence staining, after incubation with primary polyclonal antibodies against rat fibronectin (Paesel, Frankfurt, Germany) and iNOS (iNOS/NOS2) (ABR, Golden, CO, USA) and washing with TBS, the sections were incubated with Cy3-conjugated secondary antibodies (donkey antirabbit IgG-Cy3) (Dianova) for 60 minutes. After a final washing with TBS, slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Preparations were analyzed under a Zeiss Axioscope-2 microscope (Zeiss) and digital photographed using AxioVision 2 multi channel image processing system (Zeiss).

Oxidative fluorescent microtopography

The redox-sensitive fluorophore hydroethidine (Molecular Probes, Eugene, OR, USA) was used to evaluate the production of O$_2^-$ in situ as described previously [22]. Freshly cut kidney cryosections (6 μm) were placed on glass slides, topically incubated with hydroethidine (HE) (5 μmol/L) for 30 minutes. After a 30-minute incubation period, during which HE was oxidized to the fluorophore ethidium, images were obtained using a Zeiss Axioscope-2 microscope (Zeiss) and digital photographed using AxioVision 2 multi-channel image processing system (Zeiss).

EMSA

Preparation of kidney extracts and EMSA for the transcription factor NF-κB were performed as described earlier [23, 24]. Nuclear extracts (5 μg) were incubated in binding reaction medium [2 μg poly dI-dC, 1 μg bovine serum albumin (BSA), 1 mmol/L dithiothreitol (DTT), 20 mmol/L Hepes, pH 8.4, 60 mmol/L KCl, and 8% Ficoll] with 0.5 ng of $^{32}$P-deoxyadenosine triphosphate (dATP) end-labeled oligonucleotide, containing the NF-κB binding site from the major histocompatibility complex (MHC) enhancer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (H2K) (5′-gatcCAG GCC TGG GGA TTC CCC-ATC TCC ACA GG) at 30°C for 30 minutes. In competition assays 50 or 100 ng unlabeled H2K oligonucleotides were used. For supershift assay, 1 μg of anti-p50, anti-p65 (Santa Cruz Biotechnology) was added 20 minutes to the homogenates before addition of the labeled probe. For AP-1 oligonucleotides containing the consensus sequence for AP-1 (Santa Cruz Biotechnology) (5′-GAT CGA ACT GAC CGC CCG CCC GT-3′) were radiolabeled with $^{32}$P-dATP with the use of T4 polynucleotide kinase by standard methods. For AP-1 supershift assay, 1 μg of anti-c-fos, anti-c-jun, anti-Fra-1, anti-Fra-2, anti-JunB, and anti-JunD (Santa Cruz Biotechnology) was used. In competition assays 50 ng unlabeled H2K or AP-1 oligonucleotides were used.

Western blot analysis

Renal microsomal protein (15 μg per lane) was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was performed as we have described previously [17]. Protein concentrations were quantified by the Lowry method. Primary antibodies used were goat antisera against rat CYP4A1 (reacts with all rat CYP4A isoforms) and rat CYP2C11 (both Daiichi Pure Chemicals Co., Tokyo, Japan). Peroxidase-conjugated secondary antibodies were from Sigma. Blots were developed with a chemiluminescens substrate from Roche and evaluated with the Image Reader LAS-1000 (Fujifilm, Kleve, Germany).

Statistical analysis

Data are presented as mean ± SEM. Statistically significant differences in mean values were tested by analysis of variance (ANOVA), blood pressure by repeated measures ANOVA, and the Scheffé test as indicated. A value of $P < 0.05$ was considered statistically significant. The data were analyzed using Statview statistical software.

RESULTS

Seven of 15 vehicle-treated dTGR died before sacrifice. L-NIL treatment did not improve survival, as six of 15 L-NIL–treated dTGR died. EPA treatment reduced mortality to one of 15. In the combined EPA + L-NIL treatment group mortality was reduced to zero. No Sprague-Dawley rats died. The EPA effects on mortality were significant.

L-NIL–treated dTGR showed a paradoxical effect on systolic blood pressure. While blood pressures in L-NIL and L-NIL + EPA dTGR were significantly lower at week 5 compared to untreated and EPA-treated dTGR (135 ± 3 vs. 146 ± 4 vs. 167 ± 4 vs. 165 ± 6 mm Hg, respectively), blood pressure increased by 40 mm Hg within 1 week to vehicle-treated dTGR levels (Fig. 1A). However, all dTGR groups had similarly elevated blood pressures at week 7 (Fig. 1A). Cardiac hypertrophy was reduced only in the EPA-treated groups (Fig. 1B). Albuminuria was severe in vehicle-treated dTGR, but was markedly reduced in the EPA-treated groups. L-NIL alone did not reduce, but slightly increased albumin excretion (Fig. 1C).

L-NIL and EPA treatment reduced glomerular iNOS expression as shown by immunofluorescent staining (Fig. 2). Both the EPA and the L-NIL–treated groups showed reduced staining compared to vehicle treatment. Nevertheless, L-NIL treatment did not ameliorate organ damage. In the kidney, fibrosis, measured as fibronectin expression, was reduced only by EPA treatments (Fig. 3).
Fig. 1. Tail-cuff blood pressure measurements were obtained at weeks 5, 6 and 7. (A) Treatments were begun at week 4. At week 5, the L-N(6)-(1-iminoethyl) lysine (L-NIL) and the L-NIL + eicosapentanoic acid (EPA)–treated groups had lower blood pressures. At week 7, blood pressure measurements of all groups were similar. (B) Cardiac hypertrophy was partially decreased by EPA alone and EPA in combination with L-NIL. (C) Albuminuria was markedly reduced by the EPA treatment without or with L-NIL, but slightly increased with L-NIL treatment alone.
Fig. 2. Glomerular inducible nitric oxide synthase (iNOS) staining in vehicle-treated double transgenic rat (dTGR), L-N(6)-(1-iminoethyl) lysine (L-NIL)-treated dTGR, eicosapentanoic acid (EPA)-treated dTGR, EPA + L-NIL–treated dTGR, and Srague-Dawley (SD) rats. L-NIL, L-NIL + EPA, and EPA alone all reduced iNOS staining.

Fig. 3. Renal interstitial fibrosis is shown as fibronectin staining in vehicle-treated double transgenic rat (dTGR), L-N(6)-(1-iminoethyl) lysine (L-NIL)–treated dTGR, eicosapentanoic acid (EPA)-treated dTGR, EPA + L-NIL–treated dTGR, and Srague-Dawley (SD) rats.
The same was true for reactive oxygen species generation in glomeruli as shown by the fluorophore hydroethidine (Fig. 4). AP-1 activation was significantly reduced toward Sprague-Dawley control level in both EPA groups compared with vehicle-treated or L-NIL–treated dTGR (Fig. 5A). The transcription factor NF-κB was strongly activated in dTGR kidneys. This activation was partially inhibited by EPA treatment (Fig. 5B). Renal inflammation, measured as monocytes/macrophages infiltration, was decreased to Sprague-Dawley control level by EPA treatment (Fig. 5C).

As shown in Figure 6A (left panel), renal microsomes isolated from Sprague-Dawley rats converted AA to 19-/20-HETE and to a series of EETs and corresponding DHETs. None of these metabolites was produced when NADPH was omitted from the reaction mixtures. 20-HETE represented the major product of CYP4A-dependent AA hydroxylation. This metabolite migrated with a retention time of 15.8 minutes in the RP-HPLC system used. CYP2C-dependent AA epoxygenation produced 14,15-, 11,12-, and 8,9-EETs (peaks at 23.5, 24.9, and 25.3 minutes, respectively). The corresponding diols resulted from subsequent hydrolysis of the EETs catalyzed by soluble epoxide hydrolase and comprised 14,15-, 11,12-, and 8,9-DHET migrating at 12.5, 13.7, and 14.5 minutes, respectively.

The renal microsomal CYP enzymes catalyzed not only the hydroxylation and epoxygenation of AA, but also accepted EPA as an efficient alternative substrate (Fig. 6A, right panel). EPA was hydroxylated to 19-/20-OH EPA (peak at 14.2 minutes) with a rate of 453 ± 26 pmol/min/mg that clearly exceeded the corresponding AA hydroxylase activity (208 ± 12 pmol/min/mg). The EPA epoxygenase activity reached 115 ± 12 pmol/min/mg and was almost identical to the AA epoxygenase activity (108 ± 10 pmol/min/mg). However, the regional selectivities of AA and EPA epoxygenation were completely different. AA was converted to a mixture of regioisomeric EETs/DHETs and among them 11,12-EET and 11,12-DHET were the dominant products. In contrast, EPA was almost exclusively converted to 17,18-EETeTr (Fig. 6A, right panel) (peak at 19.6 minutes) demonstrating that the 17,18-double bond which distinguishes EPA from AA is a preferred site of CYP-dependent epoxygenation.

Renal microsomes isolated from dTGR and the different treatment groups produced essentially the same AA and EPA metabolite patterns as described earlier for the Sprague-Dawley controls. However, the rates of metabolite production were significantly lower in dTGR compared to the Sprague-Dawley controls, indicating a down-regulation of the CYP enzymes converting AA and EPA. AA and EPA hydroxylase activities were reduced to about 45% and the epoxygenase activities to 28% and 35% of the control values (Fig. 6B to E). Neither the hydroxylase nor the epoxygenase activities were induced by EPA, the combination EPA + L-NIL, or L-NIL treatment alone (Fig. 6B to E). Western blotting revealed a significant increase in CYP4A protein levels in both the EPA and the L-NIL treatment groups compared to vehicle-treated dTGR. The CYP4A protein level in the EPA group even exceeded that of Sprague-Dawley rats (Fig. 6F). Nevertheless, this protein increase was not correlated with an increased hydroxylase activity. In vehicle-treated dTGR, microsomal CYP2C11 protein levels reached only about 25% of the Sprague-Dawley
controls. This low level was increased to about 60% by L-NIL, but was not altered by either EPA or EPA + L-NIL treatment (Fig. 6G).

**DISCUSSION**

Our study demonstrates that EPA supplementation, but not inhibition of iNOS protects dTGR from renal damage. EPA exerted strong anti-inflammatory and antifibrotic effects and ameliorated albuminuria. L-NIL clearly inhibited the glomerular expression of iNOS, but rather worsened than ameliorated renal damage. L-NIL treatment of dTGR resulted in a strong down-regulation of glomerular iNOS expression as shown by immunohistochemistry. These findings show that L-NIL not only

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Fig. 5. Activating protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) DNA binding activity in nuclear renal extracts by electrophoretic mobility shift assay (EMSA). (A) AP-1 activation was significantly reduced by the eicosapentanoic acid (EPA) treatments. (B) NF-κB activation was partially inhibited by EPA treatment. (C) EPA suppresses monocyte/macrophage (ED-1) infiltration in the kidney. Vehicle-treated and L-N(6)-(1-iminoethyl) lysine (L-NIL)-treated double transgenic rat (dTGR) kidneys showed increased infiltration of ED-1+ cells.
functioned as an inhibitor of iNOS activity, but also simultaneously affected the regulation of iNOS expression. As a possible explanation, there are several reports indicating autoregulation of iNOS by nitric oxide itself [25]. In glomerular mesangial cells, nitric oxide seems to function in a positive feedback loop that enhances iNOS expression at the mRNA and protein level [26]. Consequently, inhibition of nitric oxide production by L-NIL can be expected to interrupt this positive feedback loop and to result in the observed reduction of iNOS protein levels.

In the dTGR model, high local Ang II concentrations trigger a cascade of events that involves oxidative stress, activation of proinflammatory transcription factors, expression of adhesion molecules, cytokines and chemokines, monocyte/macrophage infiltration, and fibrosis [18, 19, 24]. Glomerular iNOS induction is one component of this cascade. In the present study,
L-NIL effectively blocked iNOS expression as judged by immunohistochemistry. However, no end-organ protection ensued. Glomerular reactive oxygen species generation continued unabated, and we observed increased fibronectin expression and macrophage infiltration. Moreover, AP-1 and NF-κB activation were not decreased by L-NIL. In earlier studies, iNOS inhibition had variable effects indicating that enhanced nitric oxide production may have beneficial or detrimental effects depending on the pathophysiology condition [26–28]. In agreement with our study, iNOS blockade by L-NIL aggravated proteinuria in several rat models of glomerulonephritis [29–31]. These results suggest a protective role of enhanced nitric oxide production in established renal inflammation. Possibly, iNOS-derived nitric oxide may function as a feedback inhibitor of inflammatory processes by inhibiting NF-κB and AP-1 signaling [32, 33]. However, the actual mechanisms are probably more complex since nitric oxide was shown to interact with various signal transduction pathways, to counteract reactive oxygen species generation and to modulate the expression of many different genes potentially involved in glomerular remodeling [26, 34].

L-NIL treatment did not restore the low CYP-dependent AA hydroxylase and epoxygenase activities characteristic for dTGR. Down-regulation of CYP enzymes during inflammation is a general phenomenon and enhanced nitric oxide production may interfere at different levels with CYP expression and activities [35]. All CYP enzymes, including the rat renal 20-HETE and EET producing isoforms [36], can bind nitric oxide to the prosthetic heme group resulting in an inhibition of their enzymatic activities. This type of reversible CYP-nitric oxide interaction is not excluded by our findings since we determined the capacities to produce 20-HETE and EETs using isolated renal microsomes. Moreover, isoform-selective effects, including irreversible inactivation by peroxynitrite [37] and inhibition of expression by direct interaction of nitric oxide with transcription factors, have been reported [38]. Such irreversible nitric oxide effects may have contributed to the strong down-regulation of CYP2C11 protein levels in dTGR, since we observed a partial recovery after L-NIL treatment. However, our results exclude a major role of iNOS-derived nitric oxide in the irreversible down-regulation of those CYP enzymes responsible for 20-HETE (CYP4As) and EET production (predominantly CYP2C23) [17, 18]. Nitric oxide–independent down-regulation during inflammation was shown also for several other CYP isoforms but the actual mechanisms remain to be defined [35].

In contrast to L-NIL treatment, EPA supplementation clearly ameliorated Ang II–induced renal damage in our model. Numerous animal and clinical studies over the last 25 years revealed that dietary n-3 PUFAs such as EPA and docosahexaenoic acid (DHA) have beneficial effects in cardiovascular diseases [39–41]. Antiatherosclerotic, anti-inflammatory, antithrombotic, vasorelaxant, antihypertensive, and cardioprotective properties might account for the beneficial actions of EPA and DHA. The capacity of n-3 PUFAs to ameliorate renal damage is not restricted to our transgenic rat model. EPA was reported to retard the progression of diabetic nephropathy in streptozotocin-treated rats [42] and to prevent the development of glomerulosclerosis in a 5/6 renal ablation model [43]. Moreover, n-3 PUFAs delayed proteinuria, lowered blood pressure and improved the antioxidant status in spontaneously hypertensive rats [44, 45]. In our model, EPA alone or in combination with L-NIL significantly reduced albuminuria and fibronectin expression, while blood pressure was not
affected. However, EPA efficiently reduced reactive oxygen species generation, activation of AP-1 and NF-κB, and the infiltration of macrophages suggesting a strong anti-inflammatory effect. Moreover, glomerular expression of iNOS was down-regulated. The EPA effect on AP-1 and NF-κB could explain this finding since the promoter region of iNOS contains binding sites for both transcription factors [25]. Taken together, these results indicate that EPA efficiently interrupted the entire inflammatory cascade triggered by Ang II in dTGR.

The mechanisms of how n-3 PUFAs can inhibit proinflammatory transcription factor activation are not fully understood. In vitro studies suggested that EPA inhibited IκB-degradation and therefore repressed NF-κB activation [46]. On the other hand, oxidized EPA was reported to activate the peroxisome proliferator activated receptor alpha (PPAR-α), a known inhibitor of NF-κB signaling [47]. We found previously that PPAR-α activation is highly protective in our dTGR model [18]. To our knowledge, this study is the first showing inhibition of AP-1 activation by EPA in renal inflammation. In a mouse epidermal cell model, DHA and EPA inhibited AP-1 activation by a mitogen-activated protein (MAP) kinase-independent mechanism that has not yet been defined [48].

Whether the beneficial effects attributed to EPA-rich diets are exerted by EPA itself, by one of its potential metabolites, or result from reduced AA metabolite generation is unclear. EPA is known to compete with AA for the conversion by cyclooxygenases and lipooxygenases leading to a reduced production of AA-derived proinflammatory eicosanoids and to the generation of alternative less potent EPA-derived metabolites [49]. Moreover, EPA seems to be the preferred substrate for several AA-metabolizing CYP enzymes [14, 50]. Thus, a switch to CYP-dependent EPA metabolite formation may have contributed to the protective effects of EPA in our dTGR model, although EPA supplementation did not induce the CYP activities. As shown here, rat liver microsomes produce 19,20-OH-EPA and 17,18-EETeTr as the main products of EPA hydroxylation and EPA epoxygenation. The biologic activity of 17,18-EETeTr resembles that of 11,12-EET. 17,18-EETeTr acts as a powerful activator of BK channels [14]. Whether or not 17,18-EETeTr also shares the anti-inflammatory properties known for 11,12-EET is not known for certain [12].

In addition to the anti-inflammatory effects, n-3 PUFAs were reported to suppress mesangial cell proliferation [51]. EPA inhibited epidermal and platelet-derived growth factor (PDGF)-stimulated rat mesangial cell mitogenesis [52, 53]. Such effects may play a pivotal role in preventing glomerular remodeling and may also have contributed to the amelioration of renal damage in our dTGR model. Furthermore, EPA could be vasoprotective by inhibiting sphingosyl phosphorylcholine (SPC), a messenger for Rho-kinase-mediated Ca$^{2+}$-sensitization of vascular smooth muscle (VSM) contraction [54]. Further studies are required to elucidate the underlying mechanisms involved in the clinically significant antiproliferative and anti-inflammatory effects of n-3 PUFAs.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid from the Deutsche Forschungsgemeinschaft (D.N.M. and W.H.S.) and by Hoffmann-La Roche, Basel, Switzerland. J.T. was supported by a research fellowship from the Max Delbrück Center. Ms. Ramona Zummach, Ms. Christel Andricé, Ms. May-Britt Köhler, Ms Mathilde Schmidt, and Ms. Karin Dressler gave expert technical assistance.

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