Eicosapentaenoic acid restores diabetic tubular injury through regulating oxidative stress and mitochondrial apoptosis

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Taneda S, Honda K, Tomidokoro K, Uto K, Nitta K, Oda H. Eicosapentaenoic acid restores diabetic tubular injury through regulating oxidative stress and mitochondrial apoptosis. Am J Physiol Renal Physiol 299: F1451–F1461, 2010. First published September 15, 2010; doi:10.1152/ajprenal.00637.2009.—The present study was designed to elucidate a possible mechanism of hyperglycemia-induced tubular injury and to examine a therapeutic potential of dietary eicosapentaenoic acid (EPA) for the prevention of diabetic kidney disease. Utilizing streptozotocin-induced diabetic mice, the extents of albuminuria and histological injuries were monitored at 2 wk after diabetic induction. Reactive oxygen species (ROS) production, apoptosis, and hypoxia in the kidney were evaluated by immunohistochemistry and Western blotting. An in vitro study was performed using rat proximal tubular cells (NRK-52E) to confirm the protective effect of EPA for methylglyoxal (MG)-induced ROS generation and stauroporine (STS)-induced mitochondrial apoptosis. The extents of albuminuria and histological tubular injuries were significantly lower in EPA-treated diabetic mice compared with untreated diabetic mice. The levels of lipid peroxidation product (4-hydroxy-2-nonenal), oxidative DNA damage (8-hydroxy-deoxyguanosine), and mitochondrial apoptosis (TUNEL, caspase-9, cleaved caspase-3, and cytochrome c release) in the tubular cells were also significantly lower in EPA-treated diabetic mice. Furthermore, hypoxia-inducible factor (HIF)-1α expression was significantly upregulated in the kidney tissues from EPA-treated diabetic mice compared with untreated diabetic mice. MG-induced ROS overproduction and STS-induced mitochondrial apoptosis in NRK-52E cells were significantly reduced by EPA treatment in vitro. These results indicated that the ROS generation and mitochondrial apoptosis were involved in hyperglycemia-induced tubular injury and EPA had a beneficial effect by suppressing ROS generation and mitochondrial apoptosis partly through augmentation of an HIF-1α response in diabetic kidney disease.

streptozotocin; diabetic nephropathy; renal tubules; ROS; hypoxia; HIF-1α

The augmentation of reactive oxygen species (ROS) is increasingly recognized as a contributor to tubular injury in diabetes (14). Hyperglycemia leads to an increased production of ROS, such as hydrogen peroxide (H2O2) and superoxide (O2−) that may contribute to the production of advanced glycation end products (4). The overproduction of intracellular oxidative stress in response to hyperglycemia can occur in mitochondria if the intake of metabolic substrate from glucose overwhelms the mitochondrial electron transport system (8, 35). Furthermore, ROS has been shown to induce apoptosis in the proximal tubular cells of an animal model of unilateral ureteral obstruction (45). Apoptotic cells have been detected in both proximal and distal tubular epithelia of human and experimental diabetic kidneys (5, 27, 28), indicating that apoptosis might also be involved in the loss of tubular cells in diabetic nephropathy.

Eicosapentaenoic acid (EPA) is an n-3 polyunsaturated fatty acid (PUFA) that is abundant in fish oil. EPA has many effects, including antithrombotic, antihyperlipidemic (9), antiatherogenic (12), and anti-inflammatory actions (50). Recent studies have reported that EPA slows disease progression in human and experimental renal diseases including diabetic nephropathy (7, 10, 15, 16, 19, 36, 48, 49). The mechanism by which EPA ameliorates glomerular lesions is thought to involve the inhibition of thromboxan A2 (15), thromboxan B2 (49), activating protein-1 (48), monocyte chemoattractant protein-1 (19), mitogen-activated protein kinase/extracellular signal-regulated kinase signaling (19), prostaglandin E (36, 49), and prostaglandin I (49). However, little is known about the ability of EPA to protect against diabetic tubular damage, especially its effects on the molecular mechanisms involved in oxidative stress and apoptosis.

Thus we examined the mechanism of tubular injury in streptozotocin (STZ)-induced diabetic nephropathy in mice and determined whether the oral administration of EPA could prevent such lesions. Our results indicate that the overproduction of ROS and the increase in mitochondrial apoptosis in tubular epithelial cells are the key phenomena involved in diabetic tubular injury in this model and that EPA ameliorates tubular damage in response to hyperglycemia by reducing oxidative stress and mitochondrial apoptosis in the tubular epithelium.

MATERIALS AND METHODS

Study groups. Diabetes was induced in 8-wk-old female CD1 mice purchased from Charles River Laboratories (Yokohama, Japan) by the intraperitoneal injection of STZ (200 mg/kg, Sigma, Tokyo, Japan) dissolved in sterile citrate buffer (pH 4.5). STZ was administered at two time points with a 48-h interval during the first week. Twenty-four hours after STZ administration, diabetes was confirmed using the urine dip-stick method (Diasticks; Bayer Health Care, Tokyo, Japan), and the blood glucose level was determined using a Glu-test PRO R meter (n = 9). Only mice with a blood glucose level >300 mg/dl were included in the study. One day after the second STZ injection, the mice were randomly divided into three experimental study groups: 1) a group in which STZ-treated mice were fed a standard chow (n = 9), 2) a group in which STZ-treated mice were fed a standard chow supplemented with 5% EPA (wt/wt) (n = 8), and 3) a group in which STZ-treated mice were fed a standard chow and injected with insulin (Humalin-N, 1 U/day; Eli Lilly, Tokyo, Japan) once a day subcutaneously for 2 wk until death to normalize their blood glucose level (n = 6). Nondiabetic mice that were fed a standard chow (n = 6), or a standard chow supplemented with 5% EPA (wt/wt) (n = 6), were used as control groups. Body weight was examined every day until
death. Ultrapure EPA ethyl ester (>99% purity) was provided by Mochida Pharmaceutical (Tokyo, Japan). The Animal Care committee of the Tokyo Women's Medical University approved the study protocol.

Experimental design. All animals were killed at 2 wk after the induction of diabetes. Total weight of both kidneys was measured, and the kidney weight-to-body weight ratio (%) was calculated in each mouse. Six-hour urine samples were collected from each mouse 1 day before death and were assayed for urinary volume, glucose, creatinine, albuminuria, and N-acetyl-\(\beta\)-d-glucosaminidase (NAG), a marker of renal tubular damage (37) (SRL, Tokyo, Japan). A blood sample was obtained via cardiac puncture at the time of death and was assayed for creatinine and fatty acid composition (SRL). To assess renal hypoxia, pimonidazole (HPI, Burlington, MA), which binds to tissues with Po2 levels below 10 mmHg (3), was injected in vivo (50 mg/kg) 1 h before death according to the manufacturer's instructions.

Renal histology. Renal tissue was fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin according to standard protocols (1). The specimens were sectioned (2 \(\mu\)m) and stained with periodic acid methenamine-silver (silver), periodic acid-Schiff (PAS), and hematoxylin and eosin (H&E) using standard histological procedures. Tubulointerstitial injury in the cortex was analyzed histomorphometrically by counting the number of tubules that demonstrated vacuolar degeneration, chromatin condensation of tubular nuclei, and thickened and/or wrinkled tubular basement membranes divided by the number of total tubules per field \((\times 200)\) in 10 randomly selected cortical fields per cross section (33).

Immunohistochemistry. Renal tissue (4-\(\mu\)m sections) was incubated with antibodies against 8-hydroxy-deoxyguanosine [8-OHdG; clone N45.1; Japan Institute for the Control of Aging (JICA), Fukuroi, Japan], a marker for oxidative DNA damage, and 4-hydroxy-2-nonenal (4-HNE; clone HNE3-2; JICA), a marker for lipid peroxidation, as well as pimonidazole (HPI), cytochrome \(c\) (clone 7H8.2C12; BD Pharmingen, Tokyo, Japan), cleaved caspase-3 (Cell Signaling Technology, Tokyo, Japan), and caspase-9 (clone 9C3P03; Thermo Fisher Scientific Anatomical Pathology) overnight at 4°C. Then, sections were incubated with horseradish peroxidase-conjugated EnVision polymer-HRP antibody (EnVision+ polymer-HRP antibody; EnVision System; Dako Cytochemical) at 30 min. For the staining of advanced glycation end products (AGE) and glucose degradation products (GDP), paraffin-embedded sections were processed similarly and incubated with the primary antibodies against carboxymethyllysine (CM; clone 6D12, TransGenic, Tokyo, Japan), and against methylglyoxal (MG; clone 3C, NOF, Tokyo, Japan). The positive signal was detected using a tyramide signal amplification system [TSA Plus DNP (HRP) System; PerkinElmer LAS, Boston, MA] according to the manufacturer's instructions (44). Diaminobenzidine was used as a chromogen. Tissue sections were counterstained with methyl green, dehydrated, and coverslipped.

To identify immunopositive cells, the tissue sections were counterstained with PAS/methyl green for terminal transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL), 8-OHdG, and cleaved caspase-3. In each sample, the primary antibody was substituted with an irrelevant IgG from the same species and used as a negative control. The immunostaining results for cytochrome \(c\), 4-HNE, and caspase-9 were determined based on the intensity and distribution of deposition in the tubulointerstitial, as previously described (45): 0, none or trace staining; 1, <10% positive; 2, 10–30% positive; 3, 30–70% positive; 4, >70% positive in the cortex. The score was determined using 20 randomly selected, nonoverlapping fields (magnified at \(\times 200\)) in each section of the renal cortex in each mouse. The average score was calculated separately for each animal. Immunopositivity for TUNEL, cleaved caspase-3, caspase-9, and 8-OHdG in the interstitial cells or tubular cells of the cortex was counted using 20 fields, and the average number per field was determined (magnified at \(\times 100\)). For semiquantitative analysis of renal hypoxia, the number of pimonidazole-positive tubules was counted and divided by the number of total tubules per field \((\times 200)\) in 10 randomly selected cortical fields per cross section (33).

TUNEL. In situ apoptotic cell labeling was assayed using TUNEL and the Trevigen TACS 2D kit (Gaithersburg, MD) according to the manufacturer's instructions. Negative control sections, processed without terminal deoxynucleotidyltransferase, showed no staining. Trevigen-positive control slides were processed with the experimental slides and generated an apoptotic signal.

Western blot analysis. Total proteins were extracted from the whole kidney tissues or cultured cells as described previously (24). A Western blot analysis was then performed using a previously described method (47). Briefly, the membrane was first blotted with mouse monoclonal anti-hypoxia-inducible factor (HIF)-1\(\alpha\) antibody (clone H1667; Santa Cruz Biotechnology) or rabbit polyclonal caspase-9 antibody (mouse specific; Cell Signaling Technology) for proteins extracted from whole kidney tissues and with rabbit polyclonal cleaved caspase-9 (rat specific; Cell Signaling Technology) for proteins extracted from cell lysates. A chemiluminescent reagent (Pierce Biotechnology, Rockford, IL) was used to detect the products. After stripping and blocking, the membranes were reblotted with an anti-\(\gamma\)-tubulin monoclonal antibody (clone GTU-88; Santa Cruz Biotechnology). The relative expression of each protein vs. \(\gamma\)-tubulin expression was measured using National Institutes of Health Image J software.

Cell cultures. Rat proximal tubular cells (NRK-52E cells; ATCC CRL-1571) were cultured in MEM Earle's medium with 10% (vol/vol) fetal bovine calf serum containing 10 \(\mu\)M cis-5,8,11,14,17-EPA and Na salt (Sigma) for 48 h in an atmosphere of 5% CO2–95% air at 37°C. NRK-52E cells were incubated with 5 mM methylglyoxal (Sigma) for 3 h to examine the effects of glucose degradation products.

Flow cytometric analysis of ROS-producing cells and apoptotic cells. ROS production and apoptosis were analyzed using CM-2-7'-dichlorodihydrofluorescein diacetate (CM-DCF-DA; Molecular Probes) and an annexin V-FITC apoptosis detection kit (BD Biosciences), respectively. Caspase-3 activation was also evaluated by visualizing the cleavage of the PhiPhiLux-G1D2 substrate (A304R1G-3; Oncimmune) following the manufacturer's protocol. Briefly, \(5 \times 10^5\) cells were seeded into six-well tissue culture plates and incubated overnight to 60–70% confluence under standard growth conditions. For ROS production, the cells in the medium containing 10 \(\mu\)M EPA were incubated with 5 mM methylglyoxal treatment for 3 h and stained with 5 mg/ml of CM-DCF-DA for 30 min at 37°C in the dark, washed with PBS, then incubated with 7-amino-actinomycin D (7-AAD) for 15 min, and collected in 0.5 ml of PBS. To induce apoptosis, the cells were incubated with 1 \(\mu\)M staurosporine (STS; Calbiochem, Tokyo, Japan), an inducer of mitochondrial apoptosis (41), for 4 h and then stained with annexin V-FITC or caspase-3 substrate and 7-AAD for 15 min at room temperature in the dark. The samples were then analyzed using flow cytometry (BD FACS Calibur; BD Bioscience), and the resulting data were analyzed using CellQuest Pro Quick Reference version 1.1 (BD Bioscience). Each experiment was replicated twice.

Statistical analysis. All data are expressed as means \pm SE. A nonparametric Mann-Whitney U-test or a one-way ANOVA following a Tukey post hoc test were performed in statistical evaluation of the data using the SPSS program, version 13.0J for Windows (SPSS Tokyo, Japan). A \(p < 0.05\) was considered statistically significant.

RESULTS
Fatty acid composition of sera, metabolic characteristics, and renal function. An analysis of the fatty acid composition of sera revealed that EPA supplementation significantly increased the EPA concentration and decreased the arachidonic acid (AA) concentration compared with the values in the untreated groups (Table 1).
The metabolic parameters and renal function are also presented in Table 1. At the time of death, all nondiabetic mice gained body weight compared with the initial measurement. In contrast, diabetic mice except insulin-treated mice lost body weight at the time of death. The diabetic mice treated with insulin showed no significant body weight change. Both kidneys of diabetic mice were hypertrophic, and their total weight was significantly increased regardless of EPA treatment. Consequently, the kidney weight-to-body weight ratio (%) was significantly increased in the diabetic mice compared with the nondiabetic mice, but EPA had no effect on renal hypertrophy induced by diabetes. On the other hand, the diabetic mice treated with insulin showed no significant increase in their kidney weight or kidney weight-to-body weight ratio compared with the nondiabetic mice. The blood glucose levels of the diabetic mice except the insulin-treated mice were significantly elevated compared with those of the nondiabetic mice at the time of death. The daily insulin injection sufficiently reduced the blood glucose level to 101.3 ± 30.2 mg/dl in the insulin-treated diabetic mice, which was not significantly different compared with nondiabetic mice (P = 0.364). No significant differences in the blood glucose levels and body weight changes were observed between the untreated and the EPA-treated diabetic mice.

A renal function study revealed no significant difference in the serum creatinine level in any of the study groups. A significant increase in urine albumin excretion [urinary albumin excretion (µg)/creatinine (mg)] and urinary NAG excretion was detected in the untreated diabetic mice, which was reduced in EPA-treated diabetic mice (Table 1). The untreated and the EPA-treated diabetic mice showed significant increases in both urinary glucose and urine volume compared with the nondiabetic control mice, but no significant differences in these parameters were detected between the untreated and the EPA-treated diabetic mice. On the contrary, insulin treatment significantly reduced the urinary albumin excretion as well as urinary glucose, urine volume, and NAG to almost the levels of the nondiabetic mice (Table 1).

Tubular injury in diabetic mice and its amelioration by EPA. Nondiabetic mice (Fig. 1 A, and E) and EPA-treated nondiabetic mice (Supplemental Fig. S1; supplementary material for this article is available on the journal web site) demonstrated intact tubules and interstitium from the cortex to the papilla. Untreated diabetic mice uniformly developed tubulointerstitial changes (Fig. 1, B and F), demonstrating that proximal tubular epithelial cells of the cortex were finely vacuolated and contained PAS-positive materials, and the distal tubules were dilated. The proximal tubular cells occasionally exhibited chro-matin condensation of the tubular nuclei. The tubular basement membranes were slightly thickened and/or wrinkled in some tubules (Fig. 1F). These pathological findings were attenuated in the EPA-treated diabetic kidneys (Fig. 1, C and G). On the other hand, insulin-treated diabetic mice maintained in a normoglycemic condition showed no apparent tubulointerstitial alterations compared with nondiabetic mice (Fig. 1, D and H), suggesting that the etiology of tubulointerstitial injury occurred in the early phase of STZ-injected mice and was not a direct toxicity of STZ to renal tubular cells but hyperglycemia and/or glucosuria induced by STZ.

Glomerular morphology revealed no histological findings associated with diabetic glomerulosclerosis, such as an increase of mesangial matrix, microaneurysms, mesangiolysis, or nodular lesions, 2 wk after diabetic induction by STZ. Some glomeruli were slightly enlarged in the untreated diabetic (Fig. 1J) and EPA-treated diabetic mice (Fig. 1K) compared with nondiabetic (Fig. 1I) and insulin-treated diabetic mice (Fig. 1L). No apparent alteration in blood vessels was noted in any of the groups.

Quantitative analysis of tubulointerstitial damage revealed that the percentage of injured tubules in the total tubules per cortical field was significantly higher in the untreated diabetic mice (62.11 ± 2.74%) and lower in the EPA-treated diabetic mice (14.5 ± 8.4%). In insulin-treated diabetic mice, the percentage of injured tubules were small (2.3 ± 2.0%), showing no significant difference from the nondiabetic mice (0.16 ± 0.14%) (Fig. 1M).

Immunohistochemistry for AGE and GDP. Next, we performed immunohistochemistry of AGE and GDP in the untreated diabetic kidney to confirm the etiological role of hyperglycemia in the tubulointerstitial injury. Utilizing antibodies specific for carboxymethyllysine (CML) and methylglyoxal (MG), as a representative of AGE and GDP, respectively, we demonstrated the accumulation of CML and MG in the injured proximal and dilated distal tubules from the renal cortex of untreated STZ-injected diabetic mice (Fig. 2, B and E). A trace amount of immunostaining was observed in the distal tubuli of
Fig. 1. Renal histology in nondiabetic (CTR; A, E, and I), untreated diabetic [streptozotocin (STZ); B, F, and J], eicosapentaenoic acid (EPA)-treated diabetic mice (STZ+EPA; C, G, and K), and insulin-treated diabetic mice (STZ+insulin; D, H, and L). Significant tubular dilation, atrophy, and degeneration with cytoplasmic vacuolations were observed in the cortex of diabetic kidneys (STZ; B and F), and these changes were improved in the EPA-treated diabetic kidneys (STZ+EPA; C and G). Kidneys from nondiabetic mice (CTR) and insulin-treated diabetic mice (STZ+insulin) were microscopically normal. A–D: lower magnifications of the cortex (×40). E–H: higher magnifications of the cortex (×200). I–L: higher magnifications of the glomeruli (×400; periodic acid-methenamine silver stain). M: histomorphometrical analysis of injured tubules in the cortex among all study groups. The accumulation of injured tubules was observed in the cortex of untreated diabetic mice (STZ) compared with nondiabetic mice (CTR), which was decreased in EPA-treated diabetic mice (STZ+EPA). In insulin-treated diabetic mice (STZ+insulin), no significant difference was observed from the nondiabetic mice (CTR). Values are means ± SE from 6 different mice. *P < 0.01.
nondiabetic control mice (Fig. 2, A and D) and insulin-treated diabetic mice (STZ+insulin) (Fig. 2, C and F).

**Immunohistochemistry for oxidative DNA damage and lipid peroxidation.** Figure 3 demonstrates the immunostaining for 4-HNE (Fig. 3, A–C) and 8-OHdG (Fig. 3, D–F) in the tubulointerstitial cells of the renal cortex, and Table 2 shows the results of semiquantitative grading of 4-HNE staining and the number of 8-OHdG-positive cells in the tubular and interstitial cells. Oxidative DNA damage and lipid peroxidation were increased in diabetic mice and reduced by EPA treatment.

Regarding the lipid peroxidation product, nondiabetic mice showed a weak immunostaining for 4-HNE in the tubular epithelial cells of the cortex (Fig. 3A). In untreated diabetic mice, immunostaining for 4-HNE was significantly increased in the cytoplasm of the dilated distal tubules and the injured proximal tubules in the cortex (Fig. 3B); these immunostaining patterns were significantly reduced in the EPA-treated diabetic kidneys (Fig. 3C; the differences are demonstrated in Table 2). Regarding oxidative DNA damage, nondiabetic mice showed an almost negative immunostaining for 8-OHdG in the cortex (Fig. 3D). In untreated diabetic mice, a significant increase in the number of positive cells was observed in the tubular epithelial cells of the cortex (Fig. 3E); these staining patterns were significantly reduced in EPA-treated diabetic mice (Fig. 3F; the differences are demonstrated in Table 2). The immunostaining for 8-OHdG in the interstitial cells was significantly increased in the cortex of the diabetic kidneys, but no significant difference was detected between the two diabetic groups (Table 2). Insulin treatment significantly decreased the grade of 4-HNE staining and number of 8-OHdG-positive cells in the tubular and interstitial cells of the cortex to the degree of nondiabetic mice (Table 2 and Supplemental Fig. S2A and S2B).

**Fig. 2.** Accumulation of advanced glycation end product (AGE) and glucose degradation product (GDP) in the renal cortex from nondiabetic control mice (CTR; A and D), untreated diabetic mice (STZ; B and E), and insulin-treated diabetic mice (STZ+insulin; C and F), stained with antibody specific to carboxymethyllysine (CML; A–C) or methylglyoxal (MG; D–F). Positive staining for CML and MG was visualized using a tyramide signal amplification system, resulting in brown precipitates. Accumulation of CML and MG was detected in the injured proximal tubules and dilated distal tubules of untreated diabetic mice (STZ; B and E), while a trace amount of immunostaining was observed in the distal tubuli of nondiabetic mice (CTR; A and D) and insulin-treated diabetic mice (STZ+insulin; C and F). Magnification ×200 (A–F).

**Fig. 3.** Immunohistochemical analysis of 4-hydroxy-2-nonenal (4-HNE; A–C), a marker for lipid peroxidation product, and 8-hydroxy-deoxyguanosine (8-OHdG; D–F), a marker for oxidative DNA damage, in the tubulointerstitium of nondiabetic (CTR; A and D), untreated diabetic (STZ; B and E), and EPA-treated diabetic kidneys (STZ+EPA; C and F). Increased staining for 4-HNE and an increased number of 8-OHdG-positive cells were observed in untreated diabetic mice (STZ), while reductions in these parameters were observed in EPA-treated diabetic mice (STZ+EPA). Magnification: ×100 (A–C); ×200 (A–C, insets); ×200 (D–F).
was semiquantitatively graded from 0 to 4 determined by the percentage of affected area of the renal parenchyma (see MATERIALS AND METHODS).

Mitochondria-derived apoptosis of tubular epithelial cells was increased in diabetic mice and reduced by EPA. Tubular apoptosis, as detected by TUNEL or cleaved caspase-3 immunostaining, was demonstrated predominantly in the renal cortex, both in tubular and interstitial cells (Table 3). In untreated diabetic mice, TUNEL-positive cells were increased in the tubules of the cortex, compared with nondiabetic mice (Fig. 4, A and B); these findings were significantly improved in the EPA-treated diabetic mice (Fig. 4C; the differences are demonstrated in Table 3). Similarly, the number of cleaved caspase-3-positive cells was increased in the untreated diabetic mice (Fig. 4E) and reduced in the EPA-treated diabetic mice (Fig. 4F).

Immunostaining for caspase-9, a marker of mitochondrial apoptosis, was faintly detected in the cytoplasm of distal tubules but not in the proximal tubules in nondiabetic mice (Fig. 4G, Table 3); these findings were consistent with previously reported data (45). In untreated diabetic mice, the cytoplasmic caspase-9 expression was strongly increased in the distal tubules and the injured proximal tubules of the cortex (Fig. 4H); meanwhile, it was significantly reduced in the cortex of EPA-treated diabetic mice (Fig. 4I; the differences are demonstrated in Table 3). Western blot analysis of caspase-9 (49 kDa) and cleaved caspase-9 (37 kDa) using protein extracts from whole kidney tissues revealed an increase in caspase-9 (49 kDa) and cleaved caspase-9 expression (37 kDa) in untreated diabetic mice compared with those of control mice, and both products were attenuated in EPA-treated mice (Fig. 4M). A densitometric scan analysis of the Western blot revealed a 1.40- and 1.22-fold increase in the expression of caspase-9 (49-kDa product) and cleaved caspase-9 (total of both 37- and 39-kDa products), respectively, in untreated diabetic kidneys compared with the expression levels in nondiabetic kidneys (Fig. 4V). Both of these expression levels were decreased in EPA-treated kidneys compared with the levels in untreated diabetic kidneys (0.85-fold in caspase-9 and 0.73-fold in cleaved caspase-9, respectively) (Fig. 4V).

Since cytochrome c is known to be released from mitochondria into cytosol during apoptosis to activate an additional caspase pathway, cytochrome c expression in the tubular epithelium was investigated immunohistochemically. In nondiabetic mice, cytochrome c expression was easily detected especially in the distal tubular epithelial cells of the cortex, which exhibited a strong intracellular granular staining pattern, consistent with mitochondrial distribution (Fig. 4J and inset). Only a trace amount of expression was detected in the proximal tubules of the cortex (Table 3). In untreated diabetic mice, immunostaining for cytochrome c was significantly increased in both the distal tubules and the injured proximal tubular cells of the cortex, with an intracellular diffuse staining pattern (Fig. 4K and inset). These findings were improved in EPA-treated diabetic mice, which exhibited an intracellular granular staining pattern (Fig. 4L and inset; the differences are demonstrated in Table 3).

Insulin treatment significantly decreased the grades of immunostaining for TUNEL, cleaved caspase-3, caspase-9, and cytochrome c in the cortex compared with those of the untreated diabetic mice (Table 3 and Supplemental Fig. S2, C–F).

Hypoxia is associated with tubular injury and EPA-augmented HIF-1α expression in the cortex. In nondiabetic mice, pimonidazole staining was weakly detected in the medullary tubules, but not in the cortical tubules (Fig. 5A), which are normally in a state of borderline hypoxia, as previously reported (32). In contrast, the pimonidazole staining in the cortical tubules was increased in the cortex of untreated diabetic kidneys (Fig. 5B) and EPA-treated diabetic kidney (Fig. 5C). These data were consistent with the data obtained using blood oxygen level-dependent MRI (BOLD-MRI) in a previous report (38). In morphometrical analysis, the number of pimonidazole-positive tubules in the cortex was significantly increa-

Table 2. Accumulation of lipid peroxidation product and oxidative DNA damage in all study groups

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<thead>
<tr>
<th></th>
<th>CTR+ EPA</th>
<th>CTR</th>
<th>STZ</th>
<th>STZ+ EPA</th>
<th>STZ+ Insulin</th>
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<tr>
<td>4-HNE (grade)</td>
<td>1.68 ± 0.35</td>
<td>1.80 ± 0.45</td>
<td>3.33 ± 0.50*</td>
<td>2.00 ± 0.00†</td>
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<td>8-OHdG, cells/field</td>
<td>0.13 ± 0.04</td>
<td>0.11 ± 0.02</td>
<td>0.83 ± 0.42*</td>
<td>0.53 ± 0.45</td>
<td>0.10 ± 0.05†</td>
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<tr>
<td>Interstitial</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.04</td>
<td>2.69 ± 1.31*</td>
<td>1.09 ± 0.53†</td>
<td>0.05 ± 0.08†</td>
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Values are means ± SE. 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-deoxyguanosine; The degree of 4-HNE staining in the tubulointerstitial lesions was semiquantitatively graded from 0 to 4 determined by percentage of affected area of the renal parenchyma (see MATERIALS AND METHODS). *P < 0.01 vs. nondiabetic mice (CTR). †P < 0.01 vs. nontreated diabetic mice (STZ).

Table 3. Immunohistochemical analyses of TUNEL, cleaved caspase-3, caspase-9, and cytochrome c in all study groups

<table>
<thead>
<tr>
<th></th>
<th>CTR+ EPA</th>
<th>CTR</th>
<th>STZ</th>
<th>STZ+ EPA</th>
<th>STZ+ Insulin</th>
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<tr>
<td>TUNEL, cells/field</td>
<td>0.01 ± 0.02</td>
<td>0.02 ± 0.03</td>
<td>1.27 ± 0.21†</td>
<td>0.79 ± 0.39*</td>
<td>0.40 ± 0.10§</td>
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<td>Tubules</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>4.50 ± 0.69†</td>
<td>2.14 ± 0.73§</td>
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<td>Cleaved caspase-3, cells/field</td>
<td>0.00 ± 0.00</td>
<td>0.04 ± 0.01</td>
<td>1.40 ± 0.75†</td>
<td>0.85 ± 0.16†</td>
<td>0.10 ± 0.03§</td>
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<tr>
<td>Interstitial</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>3.97 ± 0.81†</td>
<td>1.40 ± 0.68†</td>
<td>0.00 ± 0.00§</td>
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<tr>
<td>Tubules</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>3.79 ± 0.39†</td>
<td>2.50 ± 1.11‡</td>
<td>1.08 ± 0.66§</td>
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<tr>
<td>Cytochrome c (grade)</td>
<td>1.23 ± 0.38</td>
<td>1.16 ± 0.41</td>
<td>2.78 ± 0.67†</td>
<td>2.00 ± 0.53‡</td>
<td>1.32 ± 0.51§</td>
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Values are means ± SE. TUNEL, terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling assay. The degree of tubulointerstitial changes expressed by caspase-9 and cytochrome c was semiquantitatively graded from 0 to 4 determined by the percentage of affected area of the renal parenchyma (see MATERIALS AND METHODS). *P < 0.05, †P < 0.01 vs. nondiabetic mice (CTR). ‡P < 0.05, §P < 0.01 vs. nontreated diabetic mice (STZ).
expression of HIF-1α was detected in nondiabetic kidneys (CTR), whereas an increased expression of HIF-1α was observed in untreated diabetic mice (1.93-fold compared with nondiabetic mice; STZ). This increase was further augmented in EPA-treated diabetic kidneys (STZ+EPA; 3.49-fold compared with nondiabetic mice and 1.81-fold compared with untreated diabetic mice) (Fig. 6, A and B).

EPA reduced ROS production and mitochondrial apoptosis in NRK-52E cells in vitro. We measured the intracellular levels of ROS in NRK-52E cells incubated with MG, a major GDP. The mean fluorescence intensity (MFI) of CM-DCF-DA in the control NRK-52E cells was 18.87 (Fig. 7A, I). After treatment with 5 mM MG for 3 h, the mean fluorescence intensity of CM-DCF-DA was increased in the untreated NRK-52E cells (109.54) (Fig. 7A, III) and significantly decreased in the EPA-treated NRK-52E cells (incubated with 10 μM EPA simultaneously) (65.26) (Fig. 7A, III) (P < 0.001; II vs. III, by Kolmogorov-Smirnov test). Next, we measured the apoptotic state of NRK-52E cells induced by STS, a potent inducer of mitochondrial apoptosis, in the absence or presence of EPA by staining the cells with annexin V and caspase-3. Regarding the annexin V expression, only 2.24% of the control cells were apoptotic (Fig. 7B, I). After treatment with 1 μM STS for 4 h, the percentage of apoptotic cells significantly increased (57.59%, MFI: 14.94) (Fig. 7B, II). In contrast, a simultaneous incubation with EPA (10 μM) significantly decreased the percentage of apoptotic cells (42.93%, MFI: 9.81) (Fig. 7B, III) (P < 0.001; II vs. III, by Kolmogorov-Smirnov test). The cell viability remained unchanged (49.52% in the cells without EPA and 52.42% in the cells with EPA).

Similarly, the MFI of the cleaved substrate of caspase-3 was increased by incubation with 1 μM STS for 4 h (25.25) (Fig. 7C, I) and was significantly decreased by the simultaneous addition of 10 μM EPA (20.52) (Fig. 7C, II) (P < 0.001; I vs. II, by Kolmogorov-Smirnov test).

Western blot analysis using protein extract from NRK-52E cells revealed the increase in cleaved caspase-9 (38 and 17 kDa) expression in NRK-52E cells by stimulation with STS (1.53-fold compared with control cells without STS), and it was reduced by the addition of EPA close to the level of the control cells (Fig. 7, D and E).
our results, we assumed that tubular injury might be a major contributor to the albuminuria during the early phase of STZ-induced diabetes in mice.

Hyperglycemia increases intracellular ROS production, nuclear DNA damage, and apoptosis through inactivation of mitochondrial enzymes (34). The involvement of subsequent mitochondrial-related mechanisms, such as activation of NADPH oxidase and mitochondrial electron transfer chain (20, 35), has also been demonstrated in tubular epithelial cells in vitro (30). These data suggest that the augmentation of ROS is increasingly recognized as a major contributor to the tubular injury in diabetes (14). In the present study, we demonstrated that EPA reduced the hyperglycemia-induced oxidative damages in the renal tubular cells in vivo and in vitro. Although the exact mechanisms responsible for the antioxidant effect of EPA are not fully understood, the antioxidant effect of EPA has been demonstrated in human and experimental renal injuries in previous reports (2, 29). In diabetes, a clinical study demonstrated that a long-term supplementation with n-3-PUFA in diabetic patients could beneficially influence the mitochondrial energy metabolism of the brain, myocardium, and pancreas and preserve these organs from later complications of diabetes mellitus (18). In addition, in some experimental settings, EPA has been shown to increase the antioxidant enzymes and genes including SOD (2), glutathione peroxidase, uncoupling protein 2, and Mn-SOD, which is generally expressed in mitochondria (29). These findings indicate that EPA may reduce ROS

DISCUSSION

The principal findings of the present study are a marked increase in oxidative stress and mitochondrial apoptosis in the renal tubular cells of STZ-induced diabetic mice, which was reduced by oral administration of EPA, ameliorating the tubular injuries and the amount of proteinuria.

Marked tubular damages, such as vacuolation of cytoplasm with pyknosis of nuclei in the renal cortex, have been reported in STZ-induced diabetic rats (43). Since apparent glomerular changes were not detected pathologically in the diabetic mice at 2 wk, the marked increase in urinary albumin excretion and the significant reduction by EPA administration can possibly be ascribed to the extent of tubular injury. A recent study demonstrated that, even with normal glomerular function, a large amount of albumin is filtered at the glomerulus and that the high amount of filtered albumin is retrieved by proximal tubular cells, which are considered to be a primary regulator of urinary protein excretion (40). This alternative view of renal albumin handling suggests that tubular injury augments urinary protein excretion by impairing reabsorption of the filtered glomerular protein, which could occur in several kinds of renal diseases accompanying tubulointerstitial injury. This theory was also supported by an in vitro study demonstrating the impairment of protein uptake in proximal tubular cells under high-glucose conditions (21) and could explain the possible mechanism of proteinuria in diabetic nephropathy. Considering

Fig. 5. Pimonidazole uptake in the kidneys from nondiabetic (CTR; A), untreated diabetic (STZ; B), and EPA-treated diabetic mice (STZ+EPA; C). Magnification: ×200 (A–C). Pimonidazole was stained only in the distal tubules of the outer stripes of the medulla in untreated diabetic mice (CTR; A). In both groups of diabetic mice, pimonidazole staining increased in the proximal tubules of the cortex, with no significant difference between the two diabetic groups; STZ and STZ+EPA (B and C). D: quantitative analysis of the percentage of pimonidazole-uptake tubules per field in the renal cortex of nondiabetic (CTR), nontreated diabetic (STZ), and EPA-treated diabetic mice (STZ+EPA). The number of pimonidazole-uptake tubules per field was counted in 10 randomly selected cortical areas (See METHODS). Results are means ± SE from 6 different mice. *P < 0.01.

Fig. 6. A: representative Western blotting for hypoxia-inducible factor (HIF)-1α using whole kidney tissues from nondiabetic (CTR), untreated diabetic (STZ), and EPA-treated diabetic mice (STZ+EPA). HIF-1α was upregulated in untreated diabetic kidney (STZ) and was further augmented in EPA-treated diabetic kidneys (STZ+EPA). B: expression levels of HIF-1α were increased in untreated diabetic mice (STZ) 1.93-fold compared with nondiabetic mice (CTR) and augmented in EPA-treated diabetic mice (STZ+EPA) 1.81-fold compared with untreated diabetic mice (STZ). Values were normalized for the differences in protein loading. Values are means ± SE from 6 different mice. *P < 0.01, #P < 0.05.
The release of cytochrome c by mitochondria pores may contribute to the release of the initiation of mitochondrial apoptosis, and the oxidation of release of cytosol in the proximal tubules of hyperglycemic mice (6). The significant reduction of ROS-derived mitochondrial apoptosis in proximal tubular epithelial cells has demonstrated in vivo and in vitro experiments. A recent study utilizing transgenic mice that selectively overexpress catalase in proximal tubular epithelial cells in our in vivo and in vitro experiments. The release of cytochrome c from mitochondria is a central event in the cytoplasm of proximal tubular cells in vivo and their injury and that EPA might have a role in regulating the mitochondrial apoptotic molecules by stabilizing mitochondrial membrane and preventing cytochrome c release and caspase activation. Furthermore, we also confirmed that EPA production via upregulation of mitochondrial regulators of hydrogen peroxide generation (46).

EPA also decreased mitochondrial apoptosis in tubular epithelial cells in our in vivo and in vitro experiments. A recent study utilizing transgenic mice that selectively overexpress catalase in proximal tubular epithelial cells has demonstrated the significant reduction of ROS-derived mitochondrial apoptosis in the proximal tubules of hyperglycemic mice (6). The release of cytochrome c from mitochondria is a central event in the initiation of mitochondrial apoptosis, and the oxidation of mitochondrial pores by ROS may contribute to the release of cytochrome c by disrupting mitochondrial membrane potential (52). The release of cytochrome c from mitochondria into the cytosol induces the activation of caspase-9 (17). Our diabetic mice also exhibited the increase in cytochrome c and caspase-9 in the cytoplasm of proximal tubular cells in vivo and their reduction by EPA. These results indicated that the mitochondria-derived apoptotic pathway is involved in diabetic tubular injury and that EPA might have a role in regulating the mitochondrial apoptotic molecules by stabilizing mitochondrial membrane and preventing cytochrome c release and caspase activation. Furthermore, we also confirmed that EPA ameliorated STS-induced mitochondrial apoptosis in cultured proximal tubular epithelial cells in vitro. STS is a strong inhibitor of protein kinase c and an inducer of mitochondrial apoptosis which has been used to induce mitochondrial apoptosis in vitro (2). Although we cannot directly connect the data collected with STS in NRK-52E cells to the diabetes-induced changes in vivo, these in vitro results are suggesting a possible role of EPA to suppress the mitochondrial apoptosis that occurred in the renal tubules of STZ-induced diabetic mice in vivo.

Hypoxia is thought to contribute to the tubular damage in diabetic kidneys (39, 51). Hyperglycemia induces osmotic diuresis (polyuria) and increases the amount of water and solutes resorption from urine. The excessive effort of tubular cells causes a relatively hypooxic state in the proximal tubular cells, and HIF-1α is elevated in the kidney at 2 wk after diabetic induction (39, 51). Consistent with previous reports (38, 39), our experimental results also showed increased hypoxic tubular cells detected by pimonidazole and upregulation of HIF-1α production in diabetic kidneys. In addition, we demonstrated that the augmented HIF-1α production in EPA-treated diabetic kidneys compared with untreated diabetic kid-
neys. Recent studies have demonstrated that O$_2^-$ reduces HIF-1α expression in renal medullary interstitial cells and that the process can be prevented by treatment with the antioxidant tempol in vitro (51). In another study, tempol also led to an increase in HIFs and heme oxygenase induction in STZ-induced diabetic kidneys, where pimonidazole was present at a similar level in vivo (39). These findings indicated that local O$_2^-$ reduces hypoxic HIF activation, probably through an increase in the proteosomal degradation of HIF (53). Considering these results, the protection of tubular damage by EPA might be mediated by augmented local HIF-1α production via suppression of intracellular oxidative stress.

HIF-1α is an important transcriptional factor that regulates protective responses to critical hypoxic injury. Candidate target genes of HIF-1α for the protection against diabetic renal tubular injury are involved in various cellular responses; such as oxygen delivery through vascular regulation (31), inhibition of apoptosis (11, 13, 23, 26), cell survival by altered glucose metabolism (42), and increased production of erythropoietin (22, 25). In a myocardial ischemia-reperfusion model, inducible nitric oxide synthase, heme oxygenase-1, and cyclooxygenase might be mediated by augmented local HIF-1α. These results, the protection of tubular damage by EPA and the increase in the proteozomal degradation of HIF (53). Considering these results, the protection of tubular damage by EPA might be mediated by augmented local HIF-1α production via suppression of intracellular oxidative stress.

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In conclusion, we have demonstrated that oral EPA administration ameliorated STZ-induced diabetic tubular injury through reduction of ROS and mitochondrial apoptosis and HIF-1α upregulation. The present study suggests that EPA can be a complementary treatment for diabetic nephropathy, particularly in patients with renal tubular injury and dysfunction.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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