Epoxide metabolites of arachidonate and docosahexaenoate function conversely in acute kidney injury involved in GSK3β signaling

Bing-Qing Denga, Ying Luoa, Xin Kanga, Chang-Bin Lia, Christophe Morisseaua,b,c, Jun Yangb,c, Kin Sing Stephen Leeb,c, Jian Huanga, Da-Yong Huab, Ming-Yu Wua, Ai Penga, Bruce D. Hammockb,c,1, and Jun-Yan Liua,b

*Center for Nephropathy and Metabolomics and Division of Nephropathy and Rheumatology, Shanghai Tenth People’s Hospital, Tongji University School of Medicine, Shanghai 200072, People’s Republic of China; bDepartment of Entomology and Nematology, University of California, Davis, CA 95616; and cComprehensive Cancer Center, University of California, Davis, CA 95616

Contributed by Bruce D. Hammock, October 12, 2017 (sent for review April 12, 2017; reviewed by Deanna L. Kroetz and Alan Parrish)

Acute kidney injury (AKI) causes severe morbidity and mortality for which new therapeutic strategies are needed. Docosahexaenoic acid (DHA), arachidonic acid (ARA), and their metabolites have various effects in kidney injury, but their molecular mechanisms are largely unknown. Here, we report that 14 (15)-epoxyeicosatrienoic acid [14 (15)-EET] and 19 (20)-epoxydocosapentaenoic acid [19 (20)-EDP], the major epoxide metabolites of ARA and DHA, respectively, have contradictory effects on kidney injury in a murine model of ischemia/reperfusion (I/R)-caused AKI. Specifically, 14 (15)-EET mitigated while 19 (20)-EDP exacerbated I/R kidney injury. Manipulation of the endogenous 19 (20)-EDP or 14 (15)-EET by alteration of their degradation or biosynthesis with selective inhibitors resulted in anticipated effects. These observations are supported by renal histological analysis, plasma levels of creatinine and urea nitrogen, and renal NGAL. The 14 (15)-EET significantly reversed the I/R-caused RTEC apoptosis and GSK3β phosphorylation in murine renal tubular epithelial cells (mRTECs), and reversed the H/R-caused reduction in GSK3β phosphorylation in mRTECs. In contrast, 19 (20)-EET dose-dependently promoted H/R-caused apoptosis and worsened the reduction in GSK3β phosphorylation in mRTECs. Overall, these epoxide metabolites of ARA and DHA function conversely in I/R-AKI, possibly through their largely different metabolic stability and their opposite effects in modulation of H/R-caused RTEC apoptosis and GSK3β phosphorylation. This study provides AKI patients with promising therapeutic strategies and clinical cautions.

Significance

This study demonstrates that 19 (20)-EDP, the major epoxide metabolite of ω-3 polysaturated fatty acid (PUFA) docosahexaenoic acid, aggravates while 14 (15)-EET, the major epoxide metabolite of ω-6 PUFA arachidonic acid, alleviates acute kidney injury (AKI) in a murine model. The metabolite 19 (20)-EDP significantly shortened while 14 (15)-EET significantly prolonged the survival of AKI mice. Opposite effects of the EDP and EET regiosiomer in ischemia/reperfusion-caused kidney injury may partially account for the opposite effects of 14 (15)-EET and 19 (20)-EDP in modulation of the hypoxia/reoxygenation-caused apoptosis of renal tubular epithelial cells and the phosphorylation of GSK3β, a promising therapeutic target for AKI. However, our study provides a caution regarding the use of dietary ω-3 fatty acids in renal injury.


Reviewer: D.L.K., University of California, San Francisco; and A.P., University of Missouri.

The authors declare no conflict of interest.

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To whom correspondence may be addressed. Email: bdhammock@ucdavis.edu or jyliu@tongji.edu.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1705615114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1705615114

PHARMACOLOGY
were accompanied by a significant reduction in phosphorylated GSK3β (p-GSK3β), resulting in an increase in GSK3β activity in vitro (17, 18). Similar changes in GSK3β and p-GSK3β were also observed in I/R- and cisplatin-caused kidney injury in animal models of AKI, and inhibition of GSK3β was found to reverse the changes and attenuate the kidney injury (17, 18). However, the role of GSK3β and its phosphorylation in the function of PUFA epoxides in kidney injury and repair has not been reported. Here, we report that the effects of the major regioisomers of EDP and EET on AKI are involved in the modulation of GSK3β phosphorylation.

Results

The 14 (15)-EET Prolonged, While 19 (20)-EDP Shortened, the Survival of I/R-Caused AKI Mice Significantly. As illustrated in Fig. 1A, 30% of the I/R-caused AKI mice survived 7 d after reperfusion, and they appeared active on day 7. However, i.p. administration of 19 (20)-EDP (100 ng every day), the most abundant regioisomer of EDPs (10, 11), to I/R-caused AKI mice significantly shortened the survival of I/R-caused AKI mice, resulting in a survival rate of 8.3% 7 d post reperfusion. In contrast, i.p. administration of 14 (15)-EET (100 ng every day) significantly prolonged the survival of I/R-caused AKI mice, resulting in a survival rate of 63.6% 7 d after reperfusion. In addition, coadministration of 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)urea (TPPU), a potent inhibitor of sEH with 14 (15)-EET or 19 (20)-EDP resulted in significantly increased survival of the EET-treated AKI mice and slightly decreased survival of the EDP-treated AKI mice, respectively.

The 14 (15)-EET Mitigated, but 19 (20)-EDP Exacerbated, the I/R-Caused Kidney Injury in Vivo. As illustrated by SI Appendix, Fig. S1, compared with the kidney of the sham mice, the kidneys of the I/R-caused AKI mice were injured, evidenced by renal tubular dilation and tubular brush border detachment. The administration of 14 (15)-EET reversed the I/R-caused dilated renal tubules toward normative status, and the coadministration of TPPU with 14 (15)-EET further enhanced the beneficial effects of treatment with 14 (15)-EET alone. In contrast, the administration of 19 (20)-EDP did not ameliorate and even worsened the I/R-caused renal injury. In addition, the coadministration of 19 (20)-EDP with TPPU resulted in wider renal tubules and more detachment of the tubular brush border than those of AKI mice, exacerbating the I/R-caused renal injury visually. In particular, the histological examination for the kidneys from the dead mice showed obviously severe injury.

As illustrated in Fig. 2A, I/R-caused AKI mice had a significantly higher plasma level of creatinine (Cr) than those of sham mice. The administration of 19 (20)-EDP to the I/R-caused AKI mice resulted in an insignificant increase in plasma Cr. The coadministration of 19 (20)-EDP with TPPU to the I/R-caused AKI mice resulted in an even higher plasma level of Cr than those of the AKI mice treated with 19 (20)-EDP alone, which was significantly higher than those of AKI mice. In contrast, the administration of 14 (15)-EET with TPPU resulted in lower plasma Cr than those of the mice treated with 14 (15)-EET alone. As expected, the plasma urea nitrogen (UN) of the mice changed following a pattern parallel to those of plasma Cr (Fig. 2B). In addition, coadministration of TPPU with 14 (15)-EET or 19 (20)-EDP significantly modified the renal level of neutrophil gelatinase-associated lipocalin (NGAL) compared with AKI mice and the AKI mice receiving 14 (15)-EET or 19 (20)-EDP alone (Fig. 2C). The renal levels of NGAL in the mice dead 24 h post treatment were much higher than those of sham mice, which were beyond the upper limit of the quantitation for the method.

TPPU Stabilized and MS-PPOH Suppressed the Epoxide Levels in Vivo. As shown in Fig. 2D and E and SI Appendix, Table S1, treatment with TPPU significantly stabilized the plasma levels of epoxides like EETs and EDPs. When TPPU was coadministered with 14 (15)-EET or 19 (20)-EDP, the plasma levels of 14 (15)-EET or 19 (20)-EDP were higher than those from dosing of 14 (15)-EET or 19 (20)-EDP alone. In contrast, when DHA was coadministered with N-(methylsulfonyl)-2-(2-propynyl)-benzenexanamidine (MS-PPOH), a potent inhibitor of PUFA epoxidation, the plasma levels of 19 (20)-EDP and EETs were lower than those of the mice treated with or without DHA (SI Appendix, Table S1).

Coadministration of MS-PPOH with DHA Enhanced the Reno-Protective Effect of DHA. As expected, the administration of DHA alone resulted in the reduction in plasma Cr (6.97 ± 0.88 vs. 9.55 ± 0.45 mM) and UN (8.9 ± 1.0 vs. 11.6 ± 0.8 mM). Coadministration
of DHA with MS-PPOH inhibited the production of EDPs, resulting in the significantly lower plasma levels of Cr (3.70 ± 1.35 μM) and UN (4.8 ± 1.8 mM) than those of the mice treated with DHA alone.

The 14 (15)-EET and 19 (20)-EDP Inhibited Inflammatory Cytokines in Vivo. As expected, I/R treatment resulted in significant increase in plasma level of TNFα, IL-6, and MCP-1 (SI Appendix, Table S2). Both coadministration of 14 (15)-EET with TPPU and co-administration of DHA with MS-PPOH significantly reduced the plasma levels of TNFα and IL-6. In addition, treatment with TPPU, 14 (15)-EET, 19 (20)-EDP, and the combination of 19 (20)-EDP with TPPU reduced the plasma level of TNFα significantly. Other treatments of the AKI mice inhibited plasma IL-6 insignificantly while all treatments were unable to modify plasma MCP-1 to the AKI mice significantly. INFγ did not change in this animal model with or without treatment.

The 14 (15)-EET Significantly Up-Regulated, While 19 (20)-EDP Negligibly Modified, the Phosphorylation of GSK3β in Vivo. As shown in Fig. 2 F–I, I/R caused a significant increase in the protein expression of GSK3β and a significant decrease in the protein expression of p-GSK3β in murine kidney, resulting in a significant decrease in the protein level ratio of p-GSK3β/GSK3β. Both dosing of 14 (15)-EET alone and co-dosing of 14 (15)-EET with TPPU significantly reversed I/R-caused decrease in protein expression of p-GSK3β, leading to the ratio of p-GSK3β/GSK3β back to a normative level. In contrast, both the administration of 19 (20)-EDP alone and the coadministration of 19 (20)-EDP with TPPU failed to modify the protein level of p-GSK3β in vivo.

The 14 (15)-EET Inhibited, While 19 (20)-EDP Promoted, the Apoptosis of Murine Renal Tubular Epithelial Cells Caused by Hypoxia/Reoxygennation Dose-Dependently. As illustrated in Fig. 3 A and F, H/R treatment caused dramatic apoptosis of murine renal tubular epithelial cells (mRTECs). Administration of 14 (15)-EET significantly inhibited the H/R-caused mRTEC apoptosis dose-dependently, while administration of 19 (20)-EDP dose-dependently promoted H/R-caused mRTEC apoptosis. Interestingly, both 14 (15)-EET and 19 (20)-EDP have negligible effect on the growth of mRTECs in normative status (SI Appendix, Fig. S2).

As expected, LiCl, a promising inhibitor of GSK3β, significantly inhibited the H/R-induced mRTEC apoptosis. Co-administration of LiCl with 14 (15)-EET or 19 (20)-EDT resulted in an additive or contradictory effect of LiCl in H/R-caused apoptosis of mRTECs (Fig. 3K). In addition, silencing RNA of GSK3β (shGSK3β) significantly inhibited, while constitutively active Ser9 of GSK3β (S9A) significantly promoted, the H/R-caused apoptosis of mRTECs compared with their respective controls. However, both 14 (15)-EET and 19 (20)-EDP failed to significantly modulate the H/R-caused apoptosis of mRTECs after the transfection with shGSK3β or S9A (Fig. 3 L and M).

The 14 (15)-EET Significantly Reversed, While 19 (20)-EDP Significantly Exacerbated, H/R-Caused Reduction in the Phosphorylation of GSK3β in mRTECs. As shown in Fig. 3 B–E, all of the treatments insignificantly modified the protein expression of GSK3β (Fig. 3 B and C). However, H/R treatment resulted in a significant decrease in the protein expression of p-GSK3β in mRTECs compared with the control group, causing a significant reduction in the ratio of p-GSK3β/GSK3β (Fig. 3 B–E). The treatment of 14 (15)-EET reversed the H/R-caused reduction in protein expression of p-GSK3β (Fig. 3 D) and the ratio of p-GSK3β/GSK3β (Fig. 3E) dose-dependently. In contrast, the administration of 19 (20)-EDP decreased the protein expression of p-GSK3β (Fig. 3F), resulting in a lower ratio of p-GSK3β/GSK3β than that of H/R-control cells dose-dependently (Fig. 3J).

The 19 (20)-EDP Is More Metabolically Stable than 14 (15)-EET. As shown in SI Appendix, Fig. S3 and Table S3, 19 (20)-EDP is more...
stable than 14 (15)-EET to hydrolysis by the murine enzyme sEH. The mouse sEH hydrolyzes 14,15-EET 24-fold faster than 19,20-EDP in vitro. This is also supported by the in vivo data in SI Appendix, Fig. S4B and Table S1; in the same treated doses, the plasma level of 19 (20)-EDP is about 10- to 15-fold higher than that of 14 (15)-EET.

Discussion

This study reports that the epoxides of ω-3 and ω-6 PUFAs have opposite effects in I/R-caused kidney injury. We first showed that the administration of 19 (20)-EDP, the abundant metabolite of the ω-3 PUFA DHA, mediated largely by CYP2C and 2J, significantly shortened the survival of the mice with I/R-caused AKI (Fig. 1A). This significantly shortened survival strongly indicates that 19 (20)-EDP aggravates the I/R-caused renal injury. This hypothesis was further supported by the histological examination of the kidneys, which clearly showed that the administration of 19 (20)-EDP alone failed in alleviating the I/R-caused renal injury (SI Appendix, Fig. S1), and coadministration of 19 (20)-EDP with TPPU that stabilized the circulation level of 19 (20)-EDP visually worsened the I/R-caused tubular dilation and detachment of brush border (SI Appendix, Fig. S1) and accelerated the death of I/R AKI mice (Fig. 1A). This hypothesis was also supported by the increases in plasma Cr and UN and renal NGAL caused by the administration of 19 (20)-EDP alone and/or the coadministration of 19 (20)-EDP with TPPU (Fig. 2A–C). In addition, the coadministration of DHA with MS-PPOH that inhibits the production of EDPs enhances the beneficial effect of DHA in the attenuation of renal injury by histological examination, plasma Cr, and UN. All these facts support that 19 (20)-EDP aggravates renal injury in the murine model of I/R-caused AKI. In contrast, administration of 14 (15)-EET, the abundant epoxide metabolite of the ω-6 PUFA ARA, significantly prolonged the survival of I/R-caused AKI mice, which strongly suggests that treatment with 14 (15)-EET reduces the I/R-caused renal injury. This hypothesis was further supported by the histological examination of the kidney, which clearly showed the improvement of tubular dilation and brush border detachment by EET treatment. The increase in plasma Cr and UN and renal NGAL caused by I/R significantly decreased following the treatment with 14 (15)-EET, which supports the hypothesis. This beneficial decrease was even enhanced by the coadministration of 14 (15)-EET with TPPU that stabilized the endogenous EETs levels. In addition, coadministration of 14 (15)-EET with TPPU significantly compressed the dramatic increase in IL-6 caused by I/R (SI Appendix, Table S2) and improved the survival of the mice with I/R AKI (Fig. 1A). All these facts support that 14 (15)-EET mitigates I/R-caused renal injury, which presents substantial proof supporting the previously reported studies that treatment with sEH inhibitors attenuates renal injury in AKI mice (13–15).
Coadministration of TPPU with 14 (15)-EET or 19 (20)-EDP further insig-"significantly modified survival over the administration of 14 (15)-EET or 19 (20)-EDP alone (Fig. 1A). This was consistent with the insignificant changes in plasma Cr (Fig. 2A) and urea (Fig. 2B) and with the insignificant changes in EETs and EDP (Fig. 2D and E and SI Appendix, Table S1) from the mice receiving coadministration of 14 (15)-EET or 19 (20)-EDP alone. However, the renal NGAL levels of mice receiving TPPU with 14 (15)-EET or 19 (20)-EDP were significantly lower or higher than those of the AKI mice treated with 14 (15)-EET or 19 (20)-EDP alone, respectively. These data support that an enhanced level of 14 (15)-EET or 19 (20)-EDP could attenuate or deteriorate kidney injury, respectively.

The epoxide metabolites of ω-3 PUFAs (e.g., EDPs) were reported sporadically regarding their biological functions although ω-3 PUFAs, such as DHA and eicosapentaenoic acid (EPA), as well as DHA- and EPA-enriched foods, have been reported and reviewed extensively to be antiinflammatory, cardio-protective, reno-protective, and tumor inhibitory (10, 19–24). Sharma et al. (12) reported recently that 19 (20)-EDP significantly reduced renal fibrosis in a murine model of unilateral ureteral obstruction (UUO)-induced uremia. The reno-protective effect of 19 (20)-EDP in a UUO murine model somewhat looks different from the results from this study. This may be ascribed to the different function of 19 (20)-EDP in renal epithelium-to-mesenchymal transition (EMT) and RTEC apoptosis, the former contributing to renal fibrosis and the latter to I/R-caused renal injury. The 19 (20)-EDP was found to significantly inhibit the renal EMT (12) but in our study significantly promoted RTEC apoptosis. Our study, together with the studies by other laboratories, reiterates the importance of studying the specific organs and/or diseases for the compounds’ function. In addition, oral administration of DHA to mice resulted in a blood level of 19 (20)-EDP similar to those from the treatment by i.p. injection (SI Appendix, Fig. S4A). Although administration of DHA results in a higher level of 19 (20)-EDP, DHA still attenuates kidney injury because DHA itself not only is renal-protective (6, 7), but also results in a higher level of renal-protective EETs in addition to 19 (20)-EDP (SI Appendix, Table S1). A pilot study of 30 healthy people in this laboratory found the serum levels of 19 (20)-EDP ranging from 0.09 to 1.2 nM. Therefore, this study also raises a caution that, extrapolating from the murine data of this study, the renal NGAL level of mice receiving DHA should be observed with care. Certainly such dietary supplementation should avoid the coadministration of sEH inhibitors and sEH inhibitor-containing foods, which may blunt the beneficial effects of DHA, or even exacerbate the renal injury.

Unlike the less studied EDPs, EETs have been extensively reported to be antiinflammatory, analgesic, vaso-validative, and cardio-protective (25–27). EETs were also regarded to be reno-protective in rodent and murine models of I/R- and cisplatin-caused AKI (13–15, 28). However, the reno-protective effect of EETs in previous studies was all conjectural based on the pharmacological intervention with either the administration of a sEH inhibitor or the targeted gene disruption of sEH (13–15, 28). Although the results are reasonable, the direct evidence followed by monitoring in vivo levels of EETs is lacking. In addition, the sEH rapidly hydrolyzes all epoxy-fatty acids tested. Therefore, blocking of sEH by pharmacological intervention with a sEH inhibitor like TPPU or target gene disruption of sEH results in the significant increase in the epoxides of many PUFAs, including but not limited to, EETs, EDPs, epoxyoctadecamonoenoic acids (epoxide metabolites of linoleic acid), and epoxyeicosatetraenoic acids (epoxide metabolites of EPA). It is difficult to distinguish EETs as reno-protective mediators from other epoxides. This study presents direct evidence that 14 (15)-EET alleviates the renal injury caused by I/R by administration of 14 (15)-EET alone. In addition, the fact that coadministration of 14 (15)-EET with TPPU stabilizes the endogenous 14 (15)-EET and enhances the reno-protection of 14 (15)-EET further testify to its reno-protective effect. This study suggests that the approaches to increasing endogenous 14 (15)-EET by treatment with 14 (15)-EET alone, and sEH inhibitors alone, as well as the combination or EET mimics are promising therapeutic and prophylactic strategies for AKI and possibly other kidney injuries.

Apoptosis of RTECs is a key step in the pathogenesis of I/R-caused renal injury (29, 30). Here, we test whether 14 (15)-EET and 19 (20)-EDP modulate the apoptosis of mRTECs in vitro in opposite ways. The mRTECs were treated with CoCl2 to cause hypoxia followed by reoxygenation to mimic I/R-caused renal injury in vivo (31, 32). The 14 (15)-EET significantly inhibited the H/R-caused mRTEC apoptosis in a positive dose-dependent manner while 19 (20)-EDP significantly promoted the H/R-caused mRTEC apoptosis dose-dependently (Fig. 3A and F). In addition, 19 (20)-EDP is more stable to sEH-mediated hydrolysis than 14 (15)-EET in vitro and in vivo (SI Appendix, Figs. S3 and S4B). These data may explain, at least in part, why 14 (15)-EET and 19 (20)-EDP have opposite effects on I/R-caused renal injury.

Administration of 14 (15)-EET with or without TPPU significantly inhibited inflammatory TNFα and IL-6, which strongly support the renal-protective effect of 14 (15)-EET. In addition, 19 (20)-EDP resulted in similar effects to 14 (15)-EET in inhibiting antiinflammatory TNFα and IL-6, consistent with the previous studies showing that 19 (20)-EDP is antiinflammatory (33). These data may indicate that the reno-toxic effect of 19 (20)-EDP is not through promoting inflammation while the antiinflammatory effect of 14 (15)-EET contributes to its reno-protective effect.

GSK3β has been found to be a key enzyme involved in kidney injury in AKI and inhibition of proliferative repair responses (16, 34). The pathogenesis of kidney injury is accompanied by a significant reduction in the phosphorylation of GSK3β and a significant increase in the activity of GSK3α (18, 35). Inhibition of GSK3β by both pharmacological interventions with chemical inhibitors and target gene deletion attenuates the kidney injury (17, 35, 36). However, the role of GSK3β’s activity in transducing the effects of epoxy fatty acids (e.g., EDPs and EETs) in kidney injury has not been established yet. We found that the protein expression of p-GSK3β in kidney tissues significantly decreased after I/R treatment, indicating an increase in the activity of GSK3β. Administration of 14 (15)-EET to AKI mice significantly up-regulated the protein expression of p-GSK3β in kidney tissues compared with those of I/R AKI mice, indicating a reduction in the activity of GSK3β. Coadministration of 14 (15)-EET with TPPU increased the circulating levels of 14 (15)-EET, but did not increase protein expression of p-GSK3β and the ratio of p-GSK3β/GSK3β compared with the mice treated with 14 (15)-EET alone. Possibly the mice treated with 14 (15)-EET alone achieved sufficient levels of EETs for the phosphorylation of GSK3β. Furthermore, in the mRTEC model, 14 (15)-EET significantly promoted the phosphorylation of GSK3α and repressed the activity of GSK3β, elucidating the inhibition of the H/R-caused apoptosis of mRTECs in vitro. In addition, coadministration of LiCl, a well-known inhibitor of GSK3β with 14 (15)-EET resulted in an additive effect in the increase in protein expression of p-GSK3β (SI Appendix, Fig. S5) and in inhibiting the H/R-caused apoptosis of mRTECs, which was consistent with administration of 14 (15)-EET to the mRTECs post transfection with shGSK3β, and constitutively active S9A failed to modulate the H/R-caused cell apoptosis significantly (Fig. 3L and M). These data strongly support that 14 (15)-EET inhibits the activity of GSK3β and contributes to its effect in reducing the apoptosis of RTECs and thus ameliorating the I/R-caused renal injury in vivo. Our finding in kidney is also consistent with previous findings that inactivation of GSK3β through increasing p-GSK3β contributes to the cardiac-protective effect of EETs (37–39). All these facts support that EETs have a protective effect on kidney injury.
effect on ischemia-caused cardiac and renal injury, which are both involved in the promotion of GSK3β phosphorylation.

In contrast, 19 (20)-EDP dose-dependently reduces the phosphorylation of GSK3β in mRTECs, consistent with its deleterious effect on H/R-caused mRTEC apoptosis in vitro and I/R-caused renal injury in vivo. However, administration of 19 (20)-EDP alone failed to decrease the phosphorylation of GSK3β in vivo. This may be due to the collective effects of the increased levels of EDP and EETs caused by treatment with 19 (20)-EDP alone (Fig. 2 D and E and SI Appendix, Table S1). In addition, co-administration of LiCl with 19 (20)-EDP to mRTECs resulted in a contradictory effect on H/R-caused apoptosis, consistent with the administration of 19 (20)-EDP to the mRTECs post transfection with shGSK3β, and constitutively active S9A failed to modulate the H/R-caused cell apoptosis significantly. These data suggest that 19 (20)-EDP induces the activity of GSK3β and contributes to its effect in promoting RTEC apoptosis and thus exacerbating the I/R-caused renal injury in vivo.

In short, this study demonstrates that the effects of epoxides of ω-3 and ω-6 PUFA s in kidney injury are the opposite: 14 (15)-EET mitigates, while 19 (20)-EDP aggravates, the I/R-caused kidney injury in a murine model. This may account, at least in part, for their opposite effects in modulation of the H/R-caused RTEC apoptosis, the phosphorylation of GSK3β, and their different metabolic stability. This study also provides AKI and other kidney disease patients with promising insights into treatments with ω-3 and ω-6 PUFA s and their epoxide metabolites for better recovery.

Materials and Methods

All animal experiments were performed according to protocols approved by the Animal Use and Care Committee of Shanghai Tenth People’s Hospital, Tongji University School of Medicine. The use of human samples was approved by the independent ethics committee of Shanghai Tenth People’s Hospital on February 29, 2016 (2016ES-91). The serum for EDP analysis was the remaining sample after clinical use from the healthy volunteers who were clinically diagnosed in the Physical Examination Department of this hospital. All of the volunteers signed an informed consent statement to approve the use of their remaining sample. Ischemia/reperfusion of kidney was conducted according to a modified protocol of the previously reported procedure (40). The group information on animal treatment is presented in Fig. 1 and SI Appendix, Table S4. The details of materials, experimental protocols, and analytical methods are presented in SI Appendix.

Acknowledgments

We thank Prof. Dr. Ya-Wei Xu (Director of Cardiovascular Disease Institute, Tongji University School of Medicine) for the use of facilities for cell culture and chemiluminescent imaging. This study was supported in part by National Natural Science Foundation of China (NSFC) Grants 11574285 and 81100090; National Institute of Environmental Health Sciences (NIEHS) Grant R01 ES02710; NIEHS Superfund Grant P42 ES04699; NIH/National Heart, Lung, and Blood Institute Grant R01 HL59699-06A1; and a Translational Technology Grant from the University of California Davis Medical Center. K.S.S.L. is supported by NIEHS Grant R00 ES024806.

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