


# A synthetic epoxyeicosatrienoic acid analogue prevents the initiation of ischemic acute kidney injury

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## Abstract

**Aim:** Imbalances in cytochrome P450 (CYP)-dependent eicosanoid formation may play a central role in ischemic acute kidney injury (AKI). We reported previously that inhibition of 20-hydroxyeicosatetraenoic acid (20-HETE) action ameliorated ischemia/reperfusion (I/R)-induced AKI in rats. Now we tested the hypothesis that enhancement of epoxyeicosatrienoic acid (EET) actions may counteract the detrimental effects of 20-HETE and prevent the initiation of AKI.

**Methods:** Male Lewis rats underwent right nephrectomy and ischemia was induced by 45 min clamping of the left renal pedicle followed by up to 48 h of reperfusion. Circulating CYP-eicosanoid profiles were compared in patients who underwent cardiac surgery with (n = 21) and without (n = 38) developing postoperative AKI.

**Results:** Ischemia induced an about eightfold increase of renal 20-HETE levels, whereas free EETs were not accumulated. To compensate for this imbalance, a synthetic 14,15-EET analogue was administered by intrarenal infusion before ischemia. The EET analogue improved renal reoxygenation as monitored by in vivo parametric MRI during the initial 2 h reperfusion phase. The EET analogue improved PI3K- as well as mTORC2-dependent rephosphorylation of Akt, induced inactivation of GSK-3 $\beta$ , reduced the development of tubular apoptosis and attenuated inflammatory cell infiltration. The EET analogue also significantly alleviated the I/R-induced drop

in creatinine clearance. Patients developing postoperative AKI featured increased preoperative 20-HETE and 8,9-EET levels.

**Conclusions:** Pharmacological interventions targeting the CYP-eicosanoid pathway could offer promising new options for AKI prevention. Individual differences in CYP-eicosanoid formation may contribute to the risk of developing AKI in clinical settings.

#### KEYWORDS

acute kidney injury, CYP-eicosanoids, inflammation, reoxygenation, signalling

## 1 | INTRODUCTION

Ischemia-reperfusion (I/R) is one of the major causes of acute kidney injury (AKI).<sup>1</sup> Ischemic AKI occurs frequently after cardiovascular surgery and kidney transplantation. Beyond acute and chronic impairment of kidney function, AKI also greatly contributes to patients' short- and long-term morbidity and mortality.<sup>2-4</sup>

Early events during the initiation phase of ischemic AKI include ATP depletion and  $\text{Ca}^{2+}$ -overload followed by activation of phospholipases A2 (PLA2).<sup>5</sup> PLA2 activation plays a critical role in I/R injury of the heart and brain,<sup>6-8</sup> however, its contribution to AKI initiation remains to be defined. PLA2 activation results in the generation of potentially toxic lyso-phospholipids as well as accumulation of free arachidonic acid (AA) that in turn may trigger disturbances in eicosanoid formation. We found that 20-hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450 (CYP)-dependent AA metabolite, is excessively released during ischemia and that inhibiting the formation or action of 20-HETE ameliorated I/R-induced AKI in rat.<sup>9</sup> Others confirmed a detrimental role of 20-HETE when using a 1-kidney model, but not in a bilateral ischemic model.<sup>10,11</sup>

In addition to 20-HETE, epoxyeicosatrienoic acids (EETs) are produced as a second class of CYP-eicosanoids throughout the renal vascular and tubular system.<sup>12-14</sup> Unlike 20-HETE, whose formation is catalyzed by CYP4A and CYP4F enzymes, EETs are produced by CYP2C and CYP2J isoforms.<sup>15,16</sup> EETs oppose the vasoconstrictor, pro-inflammatory and pro-apoptotic properties of 20-HETE.<sup>14,17,18</sup> EETs mediate vasodilator responses and represent the major endothelium-derived hyperpolarizing factor in renal arterioles.<sup>19,20</sup> EETs repress pro-inflammatory activation of endothelial cells by inhibiting cytokine-induced NF- $\kappa$ B activation and VCAM-1 expression.<sup>21</sup> EET-mediated prevention of hypoxia/reoxygenation-induced apoptosis and cell death has been demonstrated in endothelial cells and cardiomyocytes.<sup>22-24</sup> Deficiency in renal EET formation was observed in various rodent models of hypertension and target organ damage.<sup>25-28</sup> Induction or transgenic overexpression

of EET-generating CYP enzymes attenuated renal injury in rat and mouse models of angiotensin II-induced hypertension.<sup>29,30</sup> Genetic ablation of the soluble epoxide hydrolase (sEH), an enzyme mediating the degradation of EETs to dihydroxyeicosatrienoic acids (DHETs), reduced renal injury in diabetic mice.<sup>31</sup> SEH-inhibitors and most recently also metabolically robust synthetic EET analogues are under development that may provide novel therapeutic options for the treatment of cardiovascular and renal disease.<sup>32-36</sup>

In the present study, we tested the hypothesis that synthetic EET analogues may protect the kidney against I/R-induced AKI. Experiments were performed in uninephrectomized Lewis rats, a model that partially mimics the situation after renal transplantation.<sup>9</sup> The synthetic EET analogue was designed to share the functional features of the naturally occurring 11,12- and 14,15-EETs.<sup>32</sup> The animals were pretreated with the EET analogue via renal artery infusion directly before inducing ischemia. Initiation of pro-apoptotic pathways and renal reoxygenation were analysed in the early reperfusion phase. Effects on the subsequent development of renal inflammation and functional deterioration were determined two days after reperfusion.

Finally, the experimental results obtained in the rat model prompted us to speculate that individual differences in 20-HETE and EET formation may be linked to the risk of developing AKI in clinical settings. To start addressing this question, we took advantage of plasma samples collected during a recently concluded clinical trial<sup>37</sup> and compared the circulating CYP-eicosanoid profiles in patients who underwent open heart surgery with and without developing postoperative AKI.

## 2 | RESULTS

### 2.1 | Ischemia-induced imbalance between renal-free 20-HETE and EET levels

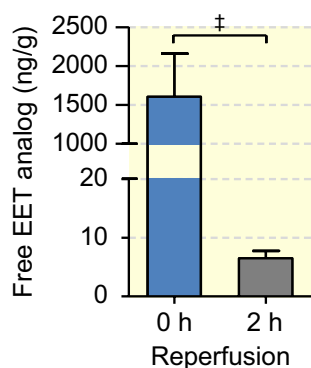
The formation of free CYP-eicosanoids in the kidney was analysed in uninephrectomized rats under the same conditions as otherwise used to induce I/R injury. The left kidneys

were pretreated with vehicle or the synthetic EET analogue and harvested either immediately at the end of the 45-min period of warm ischaemia (0 h reperfusion group) or after a subsequent 2 h reperfusion phase. The contralateral kidneys obtained during uninephrectomy before I/R served as native control. As shown in Figure 1, the EET analogue was present in the pretreated kidneys during the ischemic period in a concentration of about 1,500 ng per gram wet weight. Subsequent reperfusion resulted in a rapid washout lowering the intrarenal concentration of the EET analogue to less than 5 ng/g after 2 h.

In the native kidneys, about 96% of total 20-HETE, 98% of total EETs and 99% of total DHETs (dihydroxyeicosatrienoic acids, the products of sEH-mediated EET hydrolysis) became only detectable after alkaline hydrolysis indicating that the metabolites were predominantly esterified into membrane phospholipids under control conditions (Figure 2).

Ischemia for 45 min increased free 20-HETE levels 8-fold in the vehicle group and 11-fold in the EET analogue pretreated kidneys (Figure 2B). In contrast, free renal EET levels were not significantly higher in both groups after ischaemia compared with native controls (Figure 2D). Free DHET levels increased almost 23- and 20-fold upon ischemia, but were not significantly different comparing vehicle- and EET analogue-pretreated kidneys (Figure 2F). As a net effect, the relative abundance of free 20-HETE and free EETs was shifted from about 0.1:1 in native kidneys to 0.7:1 (vehicle group) and 1:1 (EET analogue pretreated kidneys) during 45 min of warm ischaemia. The presence of the EET analogue (compare Figure 1) had no significant effect on the ischemia-induced changes in the endogenous CYP-eicosanoid profile compared to the vehicle control.

After 2 h of reperfusion, the renal levels of free 20-HETE and DHETs strongly declined in both experimental groups



**FIGURE 1** Determination of intrarenal EET analogue levels. The EET analogue or its vehicle was infused into the left kidney 5 min before inducing ischaemia. The kidneys were harvested either immediately after 45 min of warm ischaemia (0 h reperfusion) or 2 h after reperfusion. Data are given as mean  $\pm$  SEM ( $n = 6-8$  per group). Statistically significant differences were observed as indicated: ‡( $P < .001$ )

compared to the ischemic period (Figure 2B and 2). The free EET-levels remained largely unchanged in the vehicle group, but were significantly increased by a factor of about 2.4-fold in the kidneys pretreated with the EET analogue (Figure 2D). Considering the total levels of renal CYP-eicosanoids, we observed reperfusion-induced significant elevations in esterified 20-HETE that were most pronounced in the vehicle group (Figure 2A). There were no changes in the total EET and DHET levels (Figure 2C and 2).

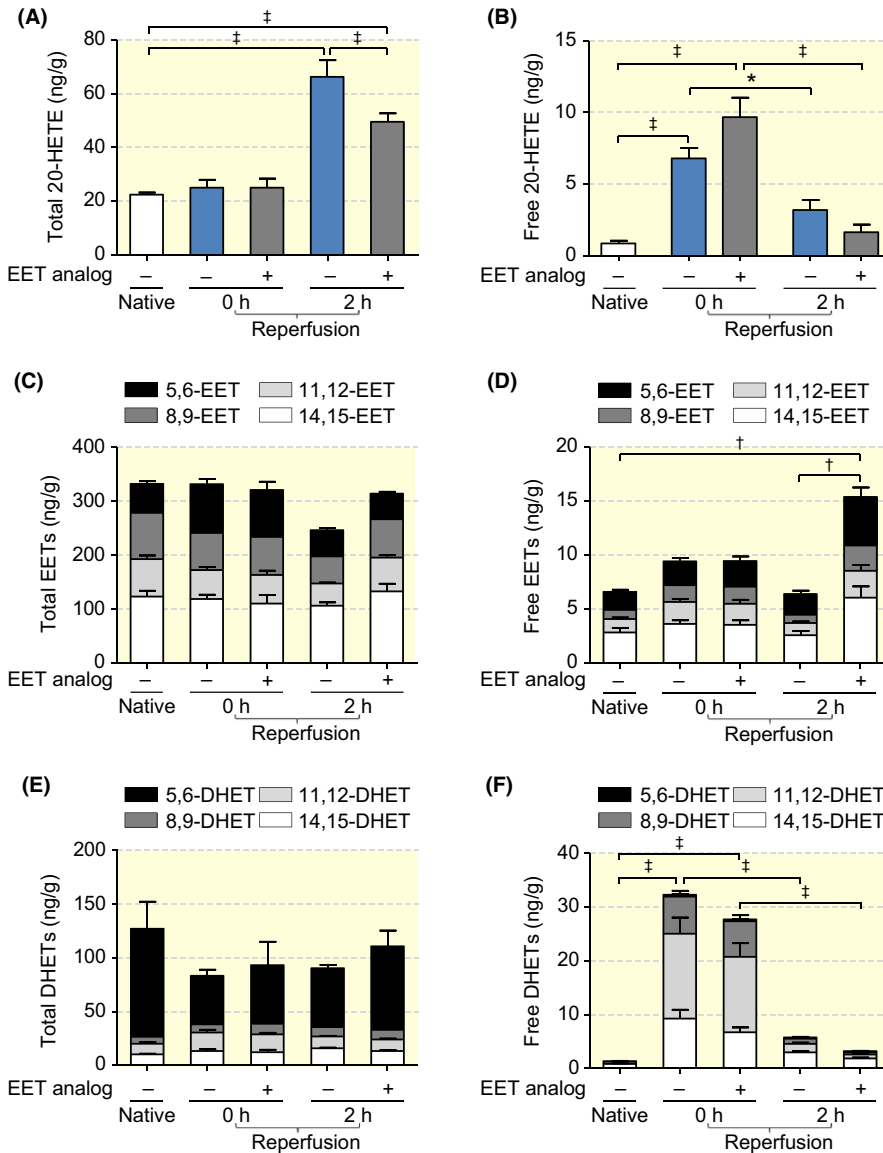
## 2.2 | EET analogue alleviates I/R-induced deterioration of renal function and tubular epithelial cell death

The EET analogue (for structural formula see Figure 3A) was administered 5 min prior to induction of ischemia via the renal artery. This single application largely preserved renal function (Figure 3B–D). Animals pretreated with the EET analogue featured significantly lower serum creatinine ( $0.61 \pm 0.06$  mg/dL vs. vehicle  $2.41 \pm 0.23$  vs. sham  $0.31 \pm 0.01$  mg/dL,  $P < .001$ ; Figure 3B) and serum urea levels ( $73.67 \pm 7.65$  mg/dL vs. vehicle  $287.80 \pm 34.81$ ,  $P < .001$  vs. sham  $37.20 \pm 1.02$  mg/dL; Figure 3C), and showed largely maintained creatinine clearance ( $1.03 \pm 0.18$  mL/min vs. vehicle  $0.26 \pm 0.02$ ,  $P < .01$ ; vs. sham  $1.37 \pm 0.04$  mL/min; Figure 3D) 48 h after reperfusion.

Pretreatment with the EET analogue also had a potent anti-apoptotic effect as shown by representative images of TUNEL staining in the outer medulla (Figure 4A and 4) and reflected by a significantly lower number of apoptotic cells obtained by semi-quantitative morphometric evaluation ( $0.26 \pm 0.02$  vs. vehicle  $4.10 \pm 0.24$  vs. sham  $0.03 \pm 0.01$  per field of view (FOV),  $P < .001$ ; Figure 4C) 48 h after reperfusion. Renal tubular damage was quantified using the acute tubular necrosis (ATN) score.<sup>38</sup> Severe damage occurred in the vehicle group as indicated by the presence of flattened tubular epithelium, exfoliated tubular epithelial cells, widened tubular lumina, hyaline cast formation and necrotic tubules. These features were markedly reduced upon pretreatment with the EET analogue (ATN score in the outer medulla:  $0.70 \pm 0.11$  vs. vehicle  $2.50 \pm 0.18$ ,  $P < .001$ ; Figure 4D). Dense infiltration with ED1-positive monocytes/macrophages was observed in the vehicle group 48 h post-ischemia indicating I/R-induced inflammation in the damaged areas of outer medulla. The EET analogue significantly repressed inflammatory cell infiltration as shown by morphometric quantification ( $11.5 \pm 2.2$  vs. vehicle  $53.4 \pm 1.5$  cells per FOV,  $P < .001$ ; Figure 4E).

## 2.3 | EET analogue improves renal reoxygenation

In an additional set of experiments, we applied a hydraulic occluder to enable remote induction of renal I/R in a 9.4

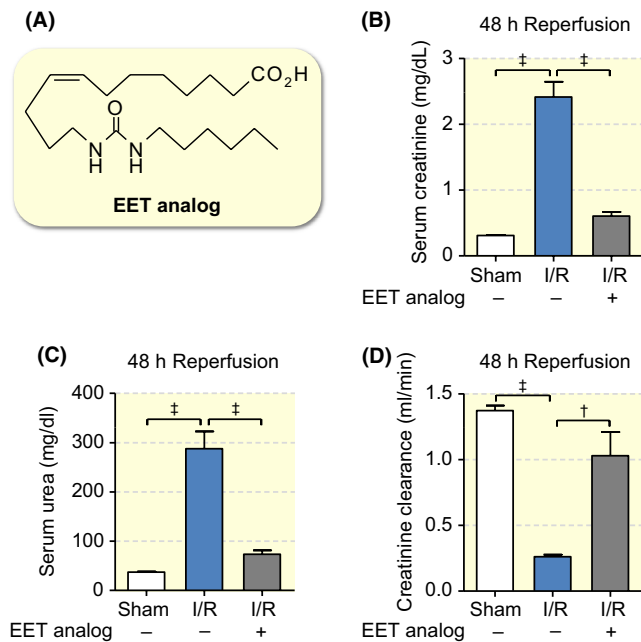


**FIGURE 2** Ischemia induces the release of 20-HETE, but not of EETs in the rat kidney. Total amounts of 20-HETE (A), EETs (C) and DHETs (E) were determined after alkaline hydrolysis of the kidney samples and represent the sum of membrane-stored (esterified into phospholipids) and free metabolites. Free metabolites (B, D and F) were extracted by treating the kidney samples with methanol without prior hydrolysis. Data are given as mean  $\pm$  SEM ( $n = 5$  per group). Statistically significant differences were observed as indicated:  $*$  ( $P < .05$ ),  $\dagger$  ( $P < .01$ ), and  $\ddagger$  ( $P < .001$ ) vs. native

Tesla small animal MR-scanner. With this setup, we performed high-spatial resolution monitoring of blood oxygenation by means of parametric magnetic resonance imaging (MRI; mapping of the relaxation time  $T_2^*$ ) during the initial I/R phase as described previously.<sup>39</sup> In the outer medulla,  $T_2^*$  remained below baseline level after reperfusion during the entire observation period of 2 h post reperfusion (Figure 5A). In contrast, cortical  $T_2^*$  exceeded baseline levels in the initial phase after reperfusion (Figure 5B). Outer medullary  $T_2^*$  levels were higher after pretreatment with the EET analogue versus vehicle, but were not restored to pre-ischemia level. Cortical  $T_2^*$  levels remained significantly elevated in the EET analogue group, whereas in vehicle-treated animals  $T_2^*$  slowly returned to baseline. Colour-coded maps showing  $T_2^*$  ratio of end-reperfusion/baseline indicate markedly improved re-oxygenation 2 h post reperfusion in renal cortex and outer medulla after treatment with the EET agonist (Figure 5D) as compared to vehicle control (Figure 5C).

## 2.4 | EET analogue contributes to induction of early pro-survival signalling

We next tested the hypothesis that the EET analogue may initiate activation of key cell survival pathways and, thereby, might restrain pro-apoptotic programming in the early I/R phase (Figures 6 and 7). To this end, kidneys were subjected to 45 min of ischemia and harvested immediately after ischemia (0h reperfusion) or after 2 h of reperfusion. Consistent with the initiation of cell protective pathways, the rephosphorylation of Akt at both Thr-308 (Figure 6A) and Ser-473 (Figure 6B) was significantly increased from 0 to 2 h of reperfusion in the EET analogue-treated kidneys, but not in the vehicle group. In native kidneys, GSK-3 $\beta$  kinase was largely inactivated by phosphorylation at Ser-9 (Figure 7A). Dephosphorylation essential for GSK-3 $\beta$  activation subsequently leading to apoptosis and inflammation was induced by ischemia. The data obtained 2 h after reperfusion showed

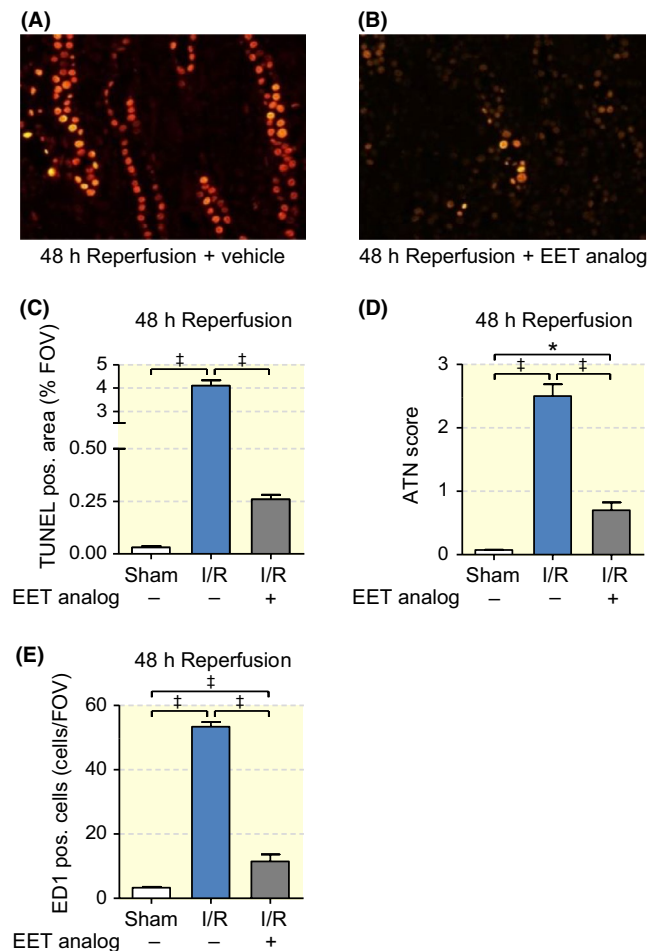


**FIGURE 3** EET analogue alleviates I/R-induced acute renal failure. Structural formula of the EET analogue (A) used in the present study. Serum creatinine (B), urea (C) and creatinine clearance (D) were determined 48 h after reperfusion: Compared to vehicle, EET analogue alleviated development of I/R induced renal function impairment. Data are given as mean  $\pm$  SEM ( $n = 5-6$  per group). Statistically significant differences were observed as indicated: †( $P < .01$ ) and ‡( $P < .001$ )

that GSK-3 $\beta$  dephosphorylation remained unchanged in the vehicle control, whereas the EET analogue mediated strong rephosphorylation up to the level of native kidneys (Figure 7A). The EET analogue also reduced the occurrence of apoptotic tubular epithelial cells in the early reperfusion phase, as revealed by TUNEL staining of kidney sections harvested 2 h after reperfusion (Figure 7B).

## 2.5 | Circulating CYP-eicosanoids in patients who do or do not develop AKI after cardiac surgery

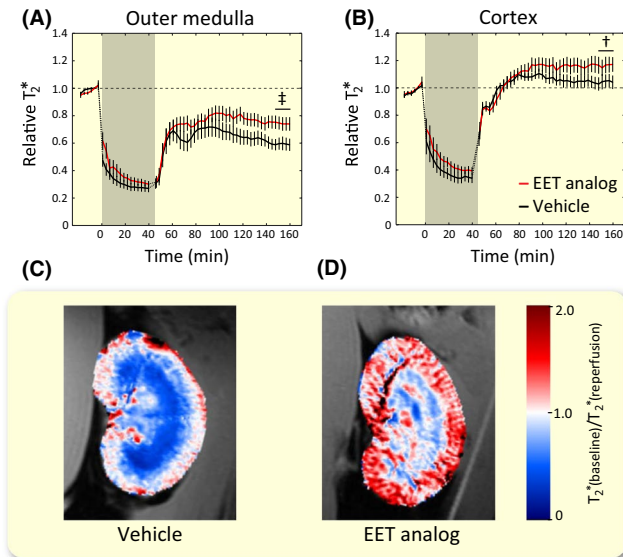
We analysed a subset of plasma samples originating from a clinical trial on the utility of sodium bicarbonate to prevent AKI in patients following open heart surgery.<sup>37</sup> This subset was derived from the non-bicarbonate-treated control arm of the study at the German Heart Centre ( $n = 81$ ) and included all available patients with postoperative AKI (AKI-group,  $n = 21$ ) as well as age- and gender-matched patients without postoperative AKI (control group;  $n = 39$ ); Table 1. The circulating CYP-eicosanoid profile was determined in plasma samples collected directly before starting the surgical intervention in order to evaluate the potential predictive value of 20-HETE and EET levels for the subsequent development of AKI. The basal 20-HETE levels were significantly higher in the AKI compared to the control group



**FIGURE 4** EET analogue alleviates I/R-induced renal epithelial cell death and intrarenal inflammation. Representative images of outer medullary sections stained by TUNEL assay (A, B) with corresponding quantitative evaluation (C), and semiquantitative acute tubular necrosis score (ATN) (D) show that EET analogue treatment significantly attenuates I/R-induced abundant epithelial apoptosis and necrosis 48 h after I/R as observed upon vehicle treatment. Quantification of monocyte/macrophage cell infiltration (ED1-positive cells/FOV) in the outer medulla (E) shows minimal ED1-positive cell infiltration upon EET analogue treatment in comparison to vehicle at 48 hours after I/R injury. Data are given as mean  $\pm$  SEM ( $n = 4-6$  per group). Statistically significant difference were observed as indicated: \*( $P < .05$ ) and ‡( $P < .001$ )

( $1.14 \pm 0.13$  vs.  $0.82 \pm 0.08$  ng/mL;  $P = .028$ ); Figure 8A. On average, patients developing postoperative AKI also showed nearly 50% higher 8,9-EET levels than controls ( $4.60 \pm 0.45$  vs.  $3.19$  ng/mL;  $P = .015$ ); Figure 8B. No significant differences were observed comparing the levels of other regioisomeric EETs in AKI and control patients (Figure 8C). Moreover, we did not detect significantly increased DHET/EET- or DiHOME/EpoME-ratios in the AKI compared to the control group indicating that differences in sEH activities were not involved in predisposing to AKI (Figure 8D and 8).





**FIGURE 5** EET analogue improves intrarenal oxygenation measured by high temporal resolution parametric MRI during the initial I/R phase. Kinetics of  $T_2^*$  changes (A, B) and color-coded  $T_2^*$  ratio maps (reperfusion/baseline) of two exemplary kidneys superimposed to the anatomical MR image (C, D) show lower extent of hypoxia and better reoxygenation after EET analogue treatment. Data are given as mean  $\pm$  SEM ( $n = 7$ -10 per group). Statistically significant differences (2-tailed  $t$  test) were observed as indicated:  $\dagger(P < .01)$  and  $\ddagger(P < .001)$  vs. vehicle

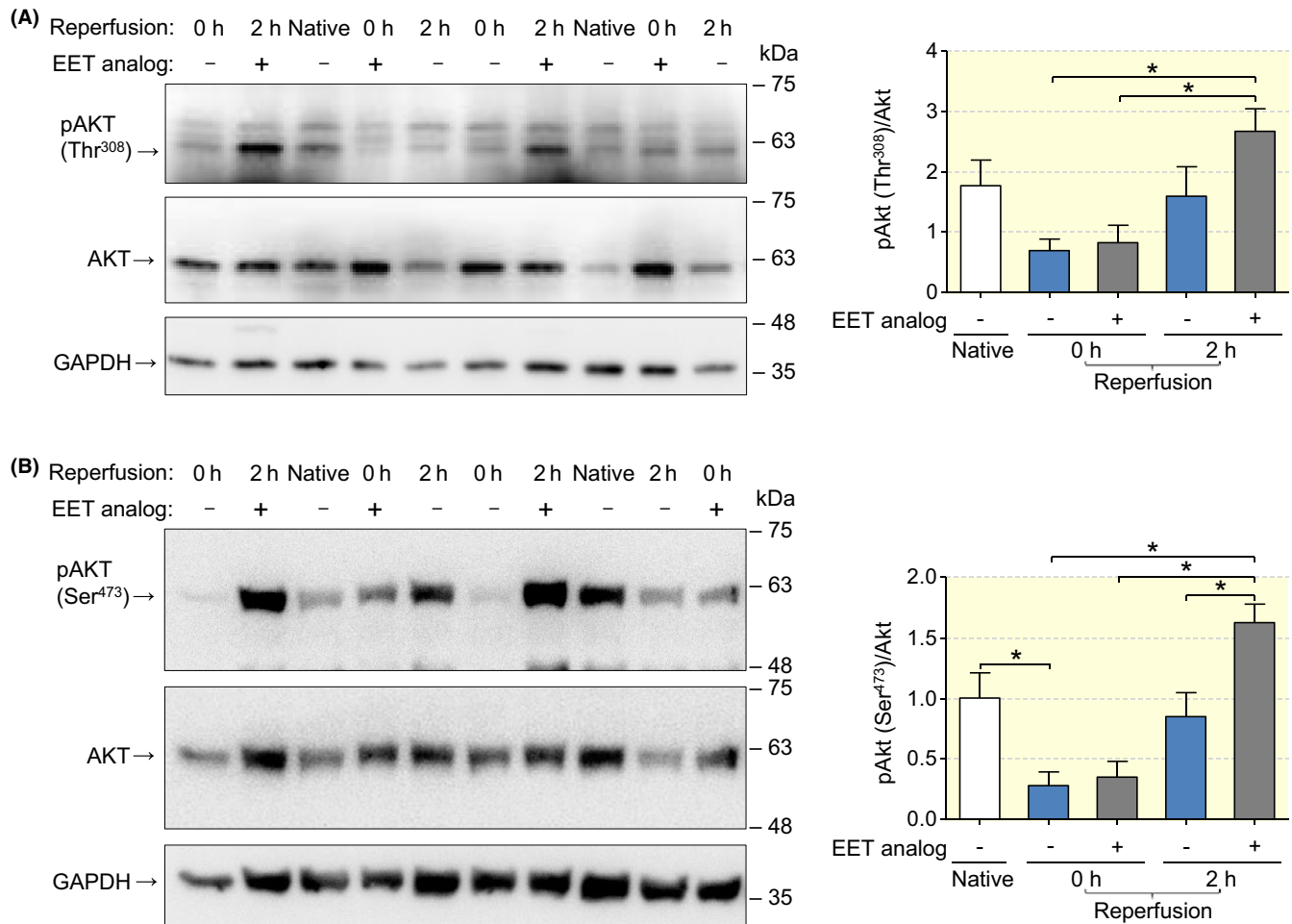
### 3 | DISCUSSION

Our experimental study shows that pretreating the kidney with a synthetic analogue of the endogenous CYP-eicosanoids 11,12- and 14,15-EET significantly alleviates I/R-induced renal injury in a rat model of AKI. Our translational pilot study indicates that individual differences in CYP-eicosanoid formation may contribute to the risk of developing AKI in patients undergoing open cardiac surgery.

Measuring the levels of free and esterified CYP-eicosanoids in the rat kidney, we found that EETs, unlike 20-HETE, did not accumulate during ischemia. Accordingly, ischemia induced an imbalance of these metabolites compared with physiological conditions. EETs and 20-HETE play opposing roles in the regulation of vascular tone, inflammation and apoptosis.<sup>14</sup> Therefore, we propose that this imbalance may contribute to the initiation of AKI. Using the same model (uninephrectomized Lewis rats), we showed previously that renal I/R-injury can be significantly ameliorated by inhibiting the generation and action of 20-HETE.<sup>9</sup> In comparison, our present data show that an even higher extent of protection can be achieved using a synthetic EET analogue with the aim of compensating for the lack of ischemia-induced endogenous EET accumulation.

EETs and 20-HETE are synthesized from free AA by CYP epoxygenases and hydroxylases respectively.<sup>13,15,40</sup> Once produced, 20-HETE and EETs are partially re-esterified into phospholipids.<sup>13,15,41</sup> This membrane pool is accessible to phospholipases activated during ischemia and, thus, provides a potential source of free 20-HETE and EETs even when de novo synthesis is limited because of hypoxia. In the kidney, ischemia induces membrane translocation and activation of  $\text{Ca}^{2+}$ -dependent phospholipase A2 (PLA2) activities.<sup>5</sup> Studies in mice deficient in cPLA2 $\alpha$  revealed that this enzyme significantly contributed to I/R-injury of the heart and brain.<sup>6-8</sup> Recently, the PLA2 inhibitor quinacrine was shown to protect against glycerol-induced AKI in rats.<sup>42</sup> Our data demonstrate that ischemia preferentially increases the renal levels of free 20-HETE and DHETs, but not EETs. A similar imbalance presumably also occurs in the ischemic heart as indicated by the different levels of these metabolites in the coronary venous plasma before and after coronary artery occlusion.<sup>43</sup> The differential accumulation of free EETs and 20-HETE may be primarily because of differences in their further metabolic fate. Free EETs are rapidly metabolized to DHETs by the sEH, an enzyme that does not require molecular oxygen and may be thus active also in the ischemic kidney.<sup>44</sup> In contrast, major routes of 20-HETE metabolism are oxygen-dependent because they are initiated by cyclooxygenases<sup>12,45</sup> and CYP enzymes.<sup>29</sup> Thus, hypoxia may limit the further metabolism of 20-HETE, but not of EETs. In the EET analogue used in this study, the labile epoxide typical of natural EETs is replaced by a metabolically and chemically more stable urea bioisostere. Furthermore, this EET analogue may have dual activity, ie, it does not only mimic the biological activities of the naturally occurring isoform (14,15-EET) but can also act as a weak sEH inhibitor as shown in vitro for the hydrolysis of an artificial substrate by the human recombinant sEH enzyme.<sup>32</sup> However, we found that the EET analogue neither affected the ischemia-induced accumulation of free DHETs and 20-HETE nor increased the levels of free EETs. Thus, it is highly unlikely that the EET analogue exerted its beneficial effects in our rat model of AKI by inhibiting the sEH.

Sustained medullary vasoconstriction that may, at least in part, be associated with endothelial dysfunction occurs in the reperfusion phase. It delays the recovery of medullary perfusion and oxygenation and, thus, contributes to I/R-induced renal injury.<sup>9,46-48</sup> Using MRI for monitoring the time course of renal blood oxygenation, we found that the EET analogue improved cortical and outer medullary reoxygenation during the early reperfusion phase. EETs mediate vasodilation of renal arterioles by stimulating calcium-activated potassium (BK) channels in vascular smooth muscle cells.<sup>19,20</sup> Moreover, EETs inhibit salt reabsorption in proximal and distal tubules by inhibiting the  $\text{Na}^+/\text{H}^+$ -exchanger and the epithelial  $\text{Na}^+$ -channel respectively.<sup>12,13,49</sup> Thus, the EET

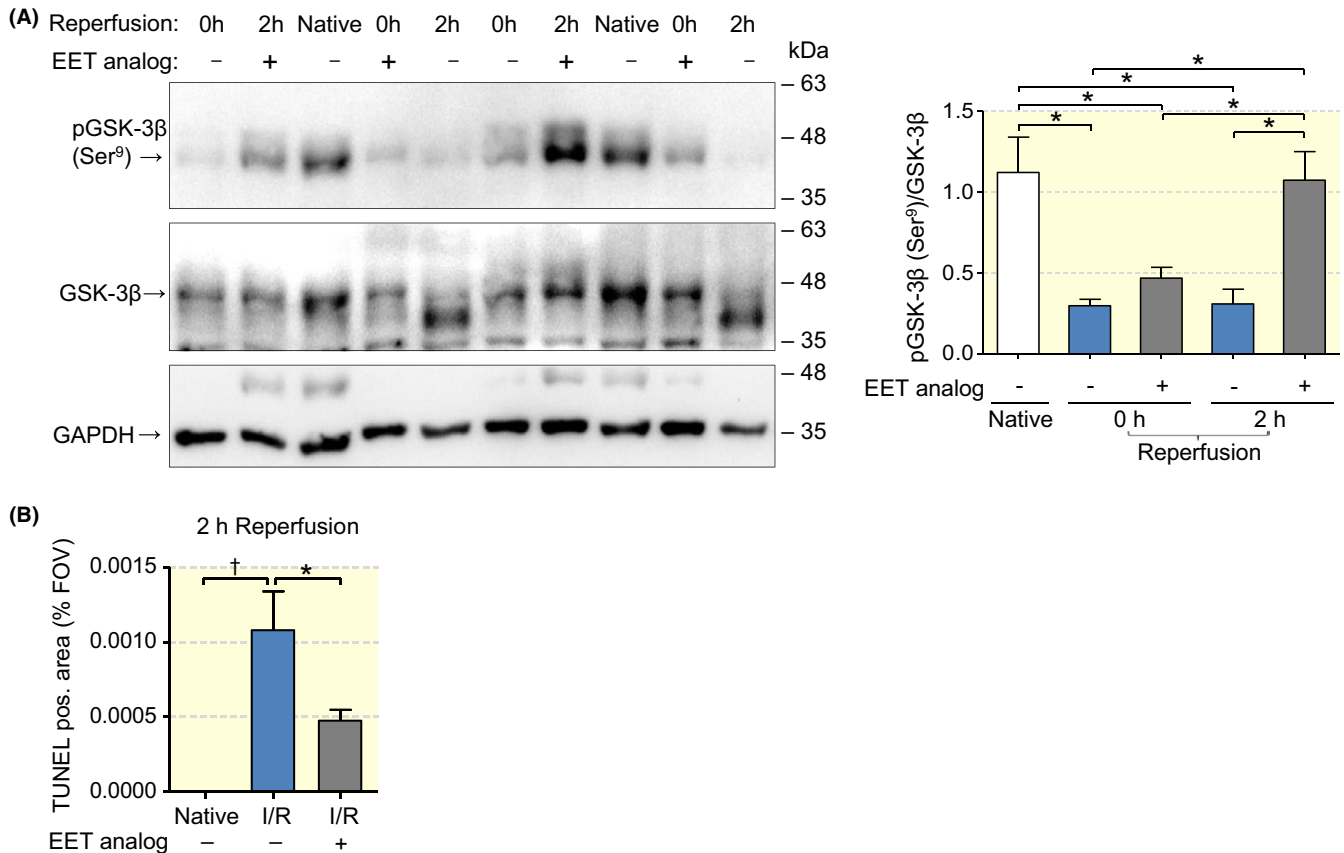


**FIGURE 6** EET analogue facilitates the restoration of Akt phosphorylation in the early reperfusion phase. Akt phosphorylation at both Thr-308 (A) and Ser-473 (B) was increased in the EET analogue compared to the vehicle group 2 h after reperfusion. Representative Western blots are shown on the left panel. Data are given as mean  $\pm$  SEM (n = 6-8 per group). Statistically significant differences were observed as indicated:  $^*(P < .05)$

analogue might have improved renal reoxygenation by both increasing microvascular oxygen supply and reducing tubular oxygen consumption. As changes in  $T_2^*$  are related to the local concentration of deoxyhemoglobin, they largely reflect variations in blood oxygenation, but may also be influenced by vasomotion and hematocrit.<sup>50</sup> EET-mediated vasodilation increases the blood volume fraction and, hence, increases renal deoxyhaemoglobin concentration (ie, decreases  $T_2^*$ ) even at unchanged blood oxygenation. The observed EET-induced increase in  $T_2^*$  might therefore underestimate the actual improvement of blood reoxygenation.

The EET analogue efficiently stimulated Akt phosphorylation at Thr-308 as well as Ser-473 indicating activation of both the PI3K and mTORC2 pathways. Activation of the PI3K/Akt survival pathway is an essential component of EET-mediated protection against apoptosis in endothelial cells and cardiomyocytes, and also plays a central role in EET-mediated protection of the heart against I/R-injury.<sup>23,24,51,52</sup> Phosphorylation of Akt at Thr-308 is required

to mediate phosphorylation and inactivation of GSK-3 $\beta$  and other pro-apoptotic mediators.<sup>53</sup> In line with this general mechanism, we found that the EET analogue markedly stimulated GSK-3 $\beta$  inactivation and reduced tubular epithelial cell apoptosis. Recently, increased GSK-3 $\beta$  phosphorylation was also shown in a mouse model of ischemic AKI after pre-treating the animals' ip with the natural metabolite 14,15-EET.<sup>54</sup> The key role of GSK-3 $\beta$  in promoting apoptosis after renal ischemia has been identified recently and substantiated by showing that direct pharmacologic inhibition of GSK-3 $\beta$  ameliorated I/R-induced tubular damage and decline of renal function.<sup>55</sup> The direct involvement of mTORC2-mediated phosphorylation of Akt at Ser-473 in renal I/R-injury has not been studied yet. Phosphorylation of Akt at Ser-473 is essential for stabilization of actin cytoskeleton<sup>56</sup> and endothelial viability in response to hypoxic stress.<sup>57</sup> Moreover, the mTORC2/Akt pathway might be related to induction of autophagy,<sup>56</sup> a mechanism increasingly recognized to protect tubular cells from degeneration and acute ischemic injury.<sup>58-60</sup>



**FIGURE 7** EET analogue facilitates the restoration of GSK-3 $\beta$  phosphorylation and acts anti-apoptotic. Lower magnitude of dephosphorylation after ischemia and successful rephosphorylation of GSK-3 $\beta$  2 h after reperfusion in the EET analogue group compared to maintained dephosphorylation of GSK-3 $\beta$  indicative of pro-apoptotic kinase activity in vehicle group (A). Quantitative evaluation of outer medullary sections stained by TUNEL assay confirming lower apoptosis rate in EET analogue-treated group (B). Data are given as mean  $\pm$  SEM ( $n = 6-8$  per group in a;  $n = 4-6$  in b). Statistically significant differences were observed as indicated: \* ( $P < .05$ ); † ( $P < .01$ )

Our data also show that the EET analogue significantly reduced I/R-induced inflammatory cell infiltration. This finding is in line with the capacity of EETs to inhibit pro-inflammatory activation of endothelial cells by repressing NF- $\kappa$ B activation.<sup>21</sup> EET-mediated anti-inflammatory effects were also shown in chronic models such as streptozotocin-induced diabetic mice<sup>31</sup> or DOCA-salt hypertensive mice.<sup>61</sup> In these studies, renal protection was achieved by pharmacological inhibition or genetic deletion of the sEH enzyme. These measures elevate endogenous EET levels through decreasing EET degradation. Compared to the extensive studies on designing and using sEH inhibitors,<sup>35,62</sup> the development of metabolically robust EET analogues feasible for in vivo application is only at the beginning.<sup>32-34,63</sup> A potential advantage of EET analogues is that they directly compensate for an EET deficiency.<sup>36</sup> In contrast, the beneficial effects of sEH inhibitors largely depend on endogenous EET production and, thus, on the expression and activity of CYP epoxygenases under the given pathological conditions. Providing first evidence for their therapeutic potential, synthetic EET analogues rescued the metabolic syndrome phenotype of HO-2-null

mice,<sup>64</sup> prevented adiposity and vascular dysfunction in rats fed a high fat diet,<sup>65</sup> attenuated cisplatin nephrotoxicity<sup>66</sup> in rats, and decreased renal inflammation and injury in ANG II hypertension.<sup>67</sup> To our knowledge, the present study is the first to demonstrate that EET analogues efficiently protect against renal I/R-injury.

Our study has several limitations related to the fact that we used a uninephrectomized model and administered the EET analogue before inducing ischaemia. Unilateral nephrectomy was shown to diminish ischemic AKI through enhanced perfusion and reduced pro-inflammatory and pro-fibrotic responses.<sup>68</sup> The preventive setting revealed a crucial role of CYP-eicosanoids in AKI initiation; however, further studies are needed to prove the therapeutic potential of EET analogues in decreasing renal injury after the ischemic event had occurred.

We believe that these findings on the role of EETs and 20-HETE in experimental renal I/R-injury might have a clinical perspective considering that AKI is a frequent complication of cardiovascular surgery and renal transplantation, ie, in clinical settings, where preventive treatment



**TABLE 1** Characteristics of patients who do or do not develop AKI after cardiac surgery

	Acute kidney injury	Control	P-value
n (total)	21	39	
Gender (f = female, m = male)	f = 4 (19%), m = 17 (81%)	f = 7 (18%), m = 32 (82%)	ns (.931)
Age (yr)	70 (52-90)	69 (50-84)	ns (.683)
Body weight (kg)	83 ± 14	82 ± 19	ns (.918)
BMI	27 ± 4	27 ± 5	ns (.642)
eGFR (pre-OP) (ml/min per 1.73 m <sup>2</sup> )	63 ± 20	68 ± 16	ns (.355)
<i>Pre-existing comorbidities</i>			
NYHA Class III-IV or EF < 35%	Yes = 4 (19%), No = 17 (81%)	Yes = 10 (26%), No = 29 (74%)	ns (.607)
Peripheral vascular disease	Yes = 4 (19%), No = 17 (81%)	Yes = 8 (21%), No = 31 (79%)	ns (.931)
Atrial fibrillation	Yes = 7 (33%), No = 14 (67%)	Yes = 14 (36%), No = 25 (64%)	ns (.897)
COPD	Yes = 4 (19%), No = 17 (81%)	Yes = 4 (10%), No = 35 (90%)	ns (.328)
Smoker	Yes = 3 (14%), No = 18 (86%)	Yes = 4 (10%), No = 35 (90%)	ns (.625)
Diabetes mell. on med.	Yes = 11 (52%), No = 10 (48%)	Yes = 28 (72%), No = 11 (28%)	ns (.179)
Insulin-dep. diabetes mell.	Yes = 0 (0%), No = 21 (100%)	Yes = 2 (5%), No = 37 (95%)	ns
Art. hypertension	Yes = 15 (71%), No = 6 (29%)	Yes = 32 (82%), No = 7 (18%)	ns (.458)
Pulmonary hypertension	Yes = 5 (24%), No = 16 (76%)	Yes = 7 (18%), No = 32 (82%)	ns (.564)
Hypercholesterolemia	Yes = 15 (71%), No = 6 (29%)	Yes = 25 (64%), No = 14 (36%)	ns (.494)
<i>Medication on admission</i>			
ACE-inhibitors/ARB	Yes = 13 (62%), No = 8 (38%)	Yes = 20 (51%), No = 19 (49%)	ns (.384)
Ca-channel-blocker	Yes = 8 (38%), No = 13 (62%)	Yes = 13 (33%), No = 26 (67%)	ns (.668)
Beta blocker	Yes = 19 (91%), No = 2 (9%)	Yes = 29 (74%), No = 10 (26%)	ns (.107)
Diuretics (pre-OP)	Yes = 14 (67%), No = 7 (33%)	Yes = 27 (69%), No = 12 (31%)	ns (.949)
Statins	Yes = 12 (57%), No = 9 (43%)	Yes = 23 (59%), No = 16 (41%)	ns (.979)
<i>Type of surgery/duration</i>			
Duration of surgery	132 ± 55	122 ± 33	ns (.358)
CABG	5	11	ns (.760)
Valve implantation	10	17	ns (.708)
Combined (CABG + Valve)	6	8	ns (.458)
Redo surgery	5	10	ns (.920)
<i>Medication during surgery</i>			

(Continues)

TABLE 1 (Continued)

	Acute kidney injury	Control	P-value
Red blood cell transfusion	no 6, yes 15	no 18, yes 29	<b>P = .042</b>
Mean transfusion Volume (ml)	1008 (250-5920)	452 (0-3830)	
Crystalloids (ml/0-24 h)	6077 ± 2067	8427 ± 1972	<b>P = .001</b>
Plasma expander (ml/0-24 h)	286 ± 435	325 ± 446	ns (.743)
Furosemide (mg/0-24 h)	82 ± 106	22 ± 23	ns (.920)
Drains (ml/0-24 h)	1006 ± 1126	609 ± 430	<b>P = .052</b>
Any inotropic (surgery day)	Yes = 14 (67%), No = 7 (33%)	Yes = 25 (64%), No = 14 (36%)	ns (.752)
Any inotropic (day 1 post OP)	Yes = 12 (57%), No = 9 (43%)	Yes = 18 (46%), No = 21 (54%)	ns (.376)
<i>Haemoglobin (g/dL)</i>			
Pre-surgery	12 ± 2	13 ± 1	ns (.274)
0 h Post surgery	11 ± 2	11 ± 2	ns (.605)
24 h Post surgery	10 ± 1	11 ± 1	<b>P = .006</b>
48 h Post surgery	11 ± 2	10 ± 3	ns (.556)

Bold values indicate statistically significant differences between the groups.

appears possible and highly desirable. Noteworthy, a renal transplantation study showed that the extent of 20-HETE release immediately following allograft reperfusion is negatively associated with post-transplant allograft function.<sup>69</sup> Furthermore, a recent candidate gene approach revealed that the risk of developing AKI following cardiac surgery is increased in patients who carry the 55Arg allele of the sEH gene that confers increased EET hydrolase activities.<sup>70</sup> Our pilot translational study suggests that increased 20-HETE and 8,9-EET levels might predispose to developing AKI following open cardiac surgery; however, proving the actual utility of these CYP eicosanoids as predictive biomarkers for AKI will now require appropriately designed prospective studies. Whereas the increased 20-HETE levels are in line with the detrimental role of 20-HETE in our unilateral model of experimental renal I/R injury,<sup>9,10</sup> the finding of increased 8,9-EET levels was rather unexpected. Among the various human CYP epoxygenases, CYP2C9 has the highest catalytic capacity of producing 8,9-EET.<sup>71</sup> Increased vascular expression of CYP2C9 has been suggested to occur in patients with coronary artery disease.<sup>72</sup> Providing a possible link between increased 8,9-EET levels and AKI, 8,9-EET can be further metabolized by COX enzymes to yield metabolites that reduce the glomerular filtration rate and may contribute to inflammatory glomerular diseases.<sup>73,74</sup>

Taken together, our results show that ischemia induces an imbalance between 20-HETE and EETs in the kidney and suggest that this imbalance plays a pivotal role in setting the stage for the cascade of events leading to renal I/R-injury. EET analogues compensating for endogenous EET deficiency in the initiation phase of renal I/R-injury may provide novel therapeutic options for the prevention of ischemic AKI.

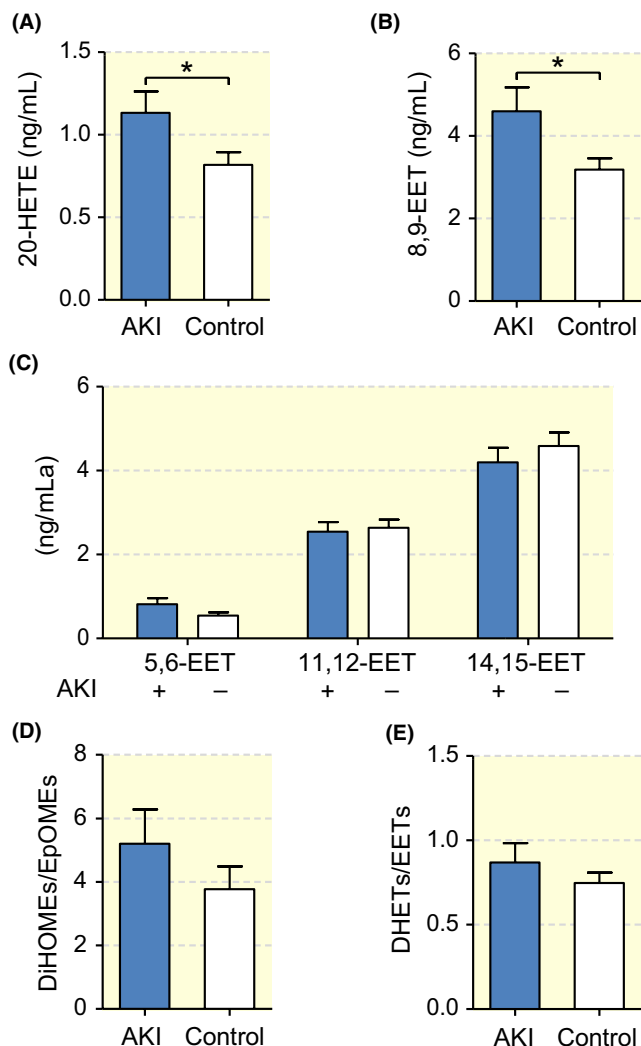
## 4 | MATERIALS AND METHODS

### 4.1 | Animals

Inbred male Lewis rats were purchased from Harlan-Winkelmann (Borchen, Germany) and housed under standard conditions. All experimental procedures were approved by the local authority (Landesamt für Gesundheit und Soziales Berlin) and were consistent with the guidelines of the Charité-Universitätsmedizin Berlin for the use of laboratory animals.

### 4.2 | Surgical procedure

Rats were 7-9 weeks of age and weighed 210-260 g. All rats (n = 5-8 per group) underwent midline laparotomy and right nephrectomy under isoflurane-induced anaesthesia. Right kidneys served as control (native group) for determination of CYP-eicosanoids and for the analysis of pro-survival signaling. EET analogue (60 µg) or its vehicle (1% DMSO in saline) was infused into the remaining left kidney by single intraaortic injection (100 µL) between two short-time aortic clamps placed above and below the level of the left renal artery. The synthetic EET analogue was synthesized as described previously.<sup>32</sup> The left kidney was exposed to 45 min of warm ischemia by clamping the renal pedicle 5 min after drug administration. During ischemia, animals were kept at a constant core body temperature (37°C ± 0.5°C). Kidneys were harvested immediately after ischemia (0 h reperfusion group) or 2 h post-reperfusion (2 h reperfusion group). Additional rats were allowed to recover (I/R + 48 h reperfusion group) and animals with uninephrectomy, but without I/R served as control (sham group). These rats received a single injection



**FIGURE 8** Circulating CYP-eicosanoids in patients who do or do not develop AKI after cardiac surgery. Shown are the pre-operational total plasma levels of 20-HETE (A), 8,9-EET (B), and other regioisomeric EETs (C) as well as the total DiHOME/ EpOME (D), and DHET/EET-ratios (E) in patients who underwent open heart surgery with ( $n = 21$ ) and without ( $n = 39$ ) developing postoperative AKI. Data are given as mean  $\pm$  SEM. Statistically significant differences ( $t$  test) were observed as indicated:  $*(P < .05)$

of buprenorphine followed by tramadol via tap water for analgesia. Rats were housed in metabolic cages for urine collection over a period of 24 h beginning one day after surgery. Rats were anaesthetized with isoflurane (2%) for final tissue sampling and termination. Blood samples were collected by puncture of the *V cava* and the remaining left kidneys were harvested 48 h post-reperfusion. The kidneys were separated, frozen in liquid nitrogen or formalin-fixed.

### 4.3 | Renal function and histology

Creatinine in serum and urine, and serum urea were measured with an automated system. Formalin-fixed and

paraffin-embedded renal sections were stained with hematoxylin and eosin to assess the severity of necrotic damage. Acute tubular necrosis (ATN) score was graded from 0 to 3 corresponding to none, mild, moderate or severe necrosis by two investigators (UH and GB) in a blinded fashion, as described previously.<sup>38</sup> Cell swelling, vacuolization, nuclear pyknosis, necrosis and cellular infiltrate were evaluated. Renal apoptosis was examined using a commercially available in situ cell death detection kit, TMR red (Roche, Grenzach-Wyhlen, Germany) following manufacturer's instructions. A minimum of 10 randomly chosen image fields of cortex and outer medulla at 400 $\times$  magnification were evaluated using AxioVision digital microscopy system (Zeiss, Jena, Germany). Positive cell staining was expressed as percentage of TUNEL-positive area per field of view (FOV). For assessment of monocyte/macrophage infiltration, tissue sections were stained with anti-ED1 antibody (Serotec, Oxford, UK) using APAAP method (Dako, Glostrup, Denmark). The number of ED1-positive cells was scored in 10 randomly chosen FOVs in cortex and outer medulla. Results were expressed as mean  $\pm$  SEM of cells per FOV at 400 $\times$  magnification. Quantitative morphometric analysis was conducted by two investigators (UH and GB) in a blinded fashion.

### 4.4 | Analysis of CYP-eicosanoids in kidney samples

Rats were prepared as described above. The left kidneys were subjected to 45 min of warm ischaemia and removed either immediately (0 h reperfusion) or after a subsequent 2-h reperfusion phase. The harvested kidneys were immediately snap-frozen in liquid nitrogen. One quarter of the kidney was pulverized in liquid nitrogen (Biopulverizer, BiospecProducts, Bartlesville, OK, USA) and 30 mg aliquots were used for subsequent analysis of CYP-eicosanoids. The pulverized samples were thawed after adding 1 mL of methanol/water (1:1) containing 2 mM ZnSO<sub>4</sub> and the mix of deuterated internal standards. ZnSO<sub>4</sub> was used to block sEH activity in the renal samples<sup>75</sup> and, thus, to protect free EETs and the corresponding internal standard from hydrolysis during the extraction procedure. Solid phase extraction was performed using Varian Bond Elut Certify II columns, either directly after adding 2 mL of 1 M sodium acetate buffer pH 6.0 to determine free metabolite levels, or after alkaline hydrolysis to determine total (free + esterified) metabolite levels as described previously.<sup>71</sup> Quantification of 20-HETE, EETs and DHETs by liquid chromatography tandem mass spectrometry (LC-MS/MS; Lipidomix GmbH, Berlin, Germany) was performed as described previously.<sup>71</sup> The EET analogue was quantified simultaneously extending the multiple reaction monitoring protocol to include the transition of its parent ion to the diagnostic fragment ion

(339.3 → 238) at a collision energy of 8 V. 10 ng of each 20-HETE-*d*6,14,15-EET-*d*8 and 14,15-DHET-*d*11 (Cayman Chemicals, Ann Arbor, MI, USA) served as internal standards and the amounts of 20-HETE, 5,6-, 8,9-, 11,12- and 14-15-EET and of the corresponding regioisomeric DHETs present in the renal samples were calculated from respective calibration curves. Results are shown as ng 20-HETE, EETs (sum of the four regioisomeric EETs) or DHETs (sum of the four regioisomeric DHETs) per g kidney weight.

#### 4.5 | Western blot analysis

The following primary antibodies were used for Western blot analyses: anti-phospho-Akt Ser473 (#4060), anti-phospho-Akt Thr308 (#2965), anti-phospho-GSK-3 $\beta$  Ser9 (#5558) and anti-total-AKT (#9272), anti-total-GSK-3 $\beta$  (#9832) from Cell Signalling Technology; GAPDH (#5G4) from HyTest (Finland). Corresponding secondary antibodies included donkey anti-rabbit HRP-IgG (#711-035-152) and donkey anti-mouse HRP-IgG (#711-035-151) from Jackson ImmunoResearch. Kidney samples (one eighth of a kidney) were homogenized in RIPA buffer supplemented with a protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitors (10 mM  $\beta$ -Glycerophosphate, 5 mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM Na-pyrophosphate). Protein samples (50  $\mu$ g of total protein) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (GE Healthcare, Amersham, UK). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-Tween) and incubated first with the primary antibodies against the phosphorylated form to be detected (1:1000 dilutions) as well as against GAPDH (1:20000) overnight at 4°C. After washing with TBS-Tween, the membranes were incubated with the appropriate secondary antibody—donkey anti-rabbit (1:15000) or donkey anti-mouse (1:25000)—for 1 h at RT. Chemiluminescent detection and image analysis were performed with ECL substrate (SuperSignal West Pico/Dura, Thermo Scientific, Rockford, IL, USA) and the G:BOX Chemi XL 1.4 imaging system (Syngene, Cambridge, UK). After visualization, the membranes were stripped using glycine-based Stripping buffer for 30 min at 52°C, and re-probed with antibodies (1:1000 dilutions) against total Akt and total GSK-3 $\beta$  respectively.

#### 4.6 | MRI monitoring of renal oxygenation

Male Lewis rats ( $n = 7$ -10 per group), aged 2-3 months, weighing 250-300 g underwent experimental I/R injury inside a 9.4 Tesla small animal MR system (Biospec 94/20, Bruker Biospin, Ettlingen, Germany) using our previously described protocol.<sup>39</sup> Briefly, the rats were anaesthetized using urethane (20% in saline, 6 mL/kg body mass) and kept at a constant core body temperature of 37°C during surgery

and MRI monitoring. Animals underwent midline laparotomy and right nephrectomy. For intrarenal administration of the EET analogue or vehicle, an aortic catheter was placed with its tip directly at the left renal branch. The rats were transferred into the MR scanner and renal blood oxygenation was continuously monitored by  $T_2^*$  mapping with a temporal resolution of ~3 min. Ischaemia was induced by closing a remotely controlled hydraulic occluder around the renal artery and vein for 45 min.<sup>39</sup> Interruption of renal blood flow was confirmed by time-of-flight (TOF) MR angiography of the kidney. Ischaemia was followed by a reperfusion phase of ~100 min.

#### 4.7 | Circulating CYP-eicosanoid levels in patients with and without the development of AKI after cardiac surgery

A subset of preoperative plasma samples collected from patients of the “Sodium Bicarbonate in Cardiac Surgery Study (Bic-MC)” (Trial registration: ClinicalTrials.gov NCT00672334) were analysed for circulating CYP-eicosanoid levels.<sup>37</sup> The study was designed as a multicentre, double-blinded (patients, clinical and research personnel), randomized controlled trial and enrolled 350 adult patients undergoing open heart surgery with the use of cardiopulmonary bypass. At induction of anaesthesia, patients received either 24 h of intravenous infusion of sodium bicarbonate (5.1 mmol/kg) or sodium chloride (5.1 mmol/kg). The primary endpoint was the proportion of patients developing acute kidney injury which was defined a priori as an increase in serum creatinine concentration greater than 25% or 0.5 mg/dL (44  $\mu$ mol/l) from baseline to peak value at any time within the first 5 d after cardiopulmonary bypass. Included were cardiac surgical patients in whom the use of cardiopulmonary bypass was planned and having one or more of the following risk factors for postoperative acute kidney injury: age above 70 years, pre-existing renal impairment (preoperative plasma creatinine concentration 120  $\mu$ mol/L), New York Heart Association class III/IV or impaired left ventricular function (left ventricular ejection fraction  $\leq$ 35%), valvular surgery or concomitant valvular and coronary artery bypass graft surgery, redo cardiac surgery, insulin-dependent Type 2 diabetes mellitus. The analysed plasma sample subset was derived from the non-bicarbonate-treated control arm of the study collected at the German Heart Centre (Berlin, Germany) ( $n = 81$ ) and included all available patients developing postoperative AKI, defined according to AKI Network criteria<sup>76</sup> (AKI-group,  $n = 21$ ) as well as age- and gender-matched patients without postoperative AKI (control group;  $n = 39$ ). The plasma levels of CYP-eicosanoids were determined after alkaline hydrolysis and solid phase extraction by LC-MS/MS as described previously.<sup>71</sup>



## 4.8 | Statistical analysis

The results were expressed as mean  $\pm$  standard error of mean (SEM). With the exception of the MRI data, statistical analysis was performed by One-way ANOVA (with Bonferroni post hoc test) or t test (if two groups were compared only) using GraphPad Prism 5 software (GraphPad Inc, La Jolla, USA).  $P < .05$  was considered significant. For MRI data,  $T_2^*$  derived from nine regions-of-interest<sup>39</sup> per kidney were statistically analysed using SPSS (version 20, IBM Deutschland GmbH, Germany). Following one-sample Kolmogorov-Smirnov test to confirm normal distribution and Levene's test to confirm equality of variances, two-tailed  $t$ -tests were performed for differences between the EET analogue group and the vehicle group, comparing the average over the last 5 time points during reperfusion. A probability value of  $\leq 0.05$  was considered significant.

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## CONFLICT OF INTEREST

The authors are not aware of conflicts of interest and have nothing to disclose.

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