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Soluble epoxide hydrolase inhibition ameliorates proteinuria-induced epithelial-mesenchymal transition by regulating the PI3K-Akt-GSK-3β signaling pathway

Yaoxian Liang [a], Ziyang Jing [a], Hui Deng [a], Zhengqian Li [b], Zhen Zhuang [a], Song Wang [a], Yue Wang [a, *]

[a] Department of Nephrology, Peking University Third Hospital, Beijing 100191, China
[b] Department of Anesthesiology, Peking University Third Hospital, Beijing 100191, China

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ABSTRACT

Soluble epoxide hydrolase (sEH) plays an essential role in chronic kidney disease by hydrolyzing renoprotective epoxyeicosatrienoic acids to the corresponding inactive dihydroxyeicosatrienoic acids. However, there have been few mechanistic studies elucidating the role of sEH in epithelial-mesenchymal transition (EMT). The present study investigated, *in vitro* and *in vivo*, the role of sEH in proteinuria-induced renal tubular EMT and the underlying signaling pathway. We report that urinary protein (UP) induced EMT in cultured NRK-52E cells, as evidenced by decreased E-cadherin expression, increased alpha-smooth muscle actin (α-SMA) expression, and the morphological conversion to a myofibroblast-like phenotype. UP incubation also resulted in upregulated sEH, activated phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB/Akt) signaling and increased phosphorylated glycogen synthase kinase-3β (GSK-3β). The PI3K inhibitor LY-294002 inhibited phosphorylation of Akt and GSK-3β as well as blocking EMT. Importantly, pharmacological inhibition of sEH with 12-(3-adamantan-1-yl- ureido)-dodecanoic acid (AUDA) markedly suppressed PI3K-Akt activation and GSK-3β phosphorylation. EMT associated E-cadherin suppression, α-SMA elevation and phenotypic transition were also attenuated by AUDA. Furthermore, in rats with chronic proteinuric renal disease, AUDA treatment inhibited PI3K-Akt activation and GSK-3β phosphorylation, while attenuating levels of EMT markers. Overall, our findings suggest that sEH inhibition ameliorates proteinuria-induced renal tubular EMT by regulating the PI3K-Akt-GSK-3β signaling pathway. Targeting sEH might be a potential strategy for the treatment of EMT and renal fibrosis.

1. Introduction

Sustained proteinuria is known to be an independent risk factor for the progression of chronic kidney disease [1]. One of the potential mechanisms of proteinuria-induced renal dysfunction is epithelial-mesenchymal transition (EMT), a process whereby epithelial cells lose their phenotypic characteristics and acquire those of mesenchymal cells [2]. Filtered proteins are taken up by tubular cells leading to tubular synthesis of various proinflammatory and profibrogenic cytokines. Proteinuria is recognized as one of the major inducers of EMT that contributes to the development of renal fibrosis [3–5].

Epoxyeicosatrienoic acids (EETs) are cytochrome P-450 metabolites of arachidonic acid with potent anti-inflammatory and antifibrotic effects [6,7]. Once formed, EETs are rapidly converted in the cytosol, by soluble epoxide hydrolase (sEH), to their corresponding less potent diols known as dihydroxyeicosatrienoic acids (DHETs) [8]. sEH is highly enriched in the renal proximal tubular epithelial cells. Pharmacological inhibition or genetic disruption of sEH increases accumulation of EETs and exerts protective effects in

Abbreviations: α-SMA, alpha-smooth muscle actin; ADR, Adriamycin; AUDA, 12-(3-adamantan-1-yl-ureido)- dodecanoic acid; EETs, epoxyeicosatrienoic acids; EMT, epithelial-mesenchymal transition; GSK-3β, glycogen synthase kinase-3β; PI3K, phosphatidylinositol 3-kinase; sEH, soluble epoxide hydrolase; TGF-β, transforming growth factor-beta; UP, urinary protein.

* Corresponding author. Department of Nephrology, Peking University Third Hospital, No. 49, North Garden Road, Haidian District, Beijing 100191, China. Fax: +86 10 8226 6727.
E-mail address: bjwangyue@sina.com (Y. Wang).

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diabetic nephropathy and hypertensive renal damage [9,10]. We previously reported that the upregulation of sEH in proximal tubular cells mediated proteinuria-induced renal damage [3], but the underlying molecular mechanism has remained elusive.

The phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB/Akt)-glycogen synthase kinase-3 beta (GSK-3β) pathway plays an important role in regulating growth, proliferation, survival, metabolism, and other cellular activities [11]. Accumulating evidence indicates that the PI3K-Akt-GSK-3β pathway is an important contributor to EMT and fibrosis [12–14]. Additionally, sEH inhibition has anti-apoptotic effects via the PI3K-Akt pathway in kidney and brain [10,15], suggesting a close association between sEH and the PI3K-Akt signaling pathway.

The goal of our present study was to address the potential role and underlying mechanism of sEH in the pathogenesis of proteinuria-induced renal proximal tubular EMT. We hypothesized that pharmacological inhibition of sEH would prevent EMT by regulating the PI3K-Akt-GSK-3β pathway both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Extraction of urinary protein

The study protocol was approved by the Peking University Biomedical Ethics Committee (Beijing, China) and the informed consents were obtained. Urine samples were collected from patients with minimal change disease who had not received any treatment. Urinary protein (UP) was isolated and purified by ammonium sulfate precipitation. The extracted protein liquid was lyophilized to a powder and dissolved in cell culture medium. Endotoxin was removed using polymyxin B-immobilized fiber columns. Finally, the protein solution was filtered through a 0.22-μm pore size filter.

2.2. Cell culture

Rat renal proximal tubular epithelial cells (NRK-52E, Peking Union Medical College, Beijing, China) were cultured in DMEM high glucose medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) in a humidified environment with 5% CO_2 at 37 °C. NRK52E cells were cultured in medium containing 0.1% FBS overnight before treatment with 10 mg/ml UP for 48 h. Cells were treated with pharmacological inhibitors before receiving UP, as follows: PI3K inhibitor LY-294002 (Sigma, St. Louis, MO, USA) 25 μM for 30 min; sEH inhibitor 12-(3-adamantan-1-yl- ureido)-dodecanoic acid (AUDA, Sigma) 1 μM for 30 min.

2.3. Animal experiments

All animal experiments were conducted in accordance with protocols approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Beijing, China). Male Wistar rats (Department of Laboratory Animal Science, Peking University Health Science Centre, Beijing, China), weighing 200–250 g, were bred and maintained under standardized housing conditions with food and water *ad libitum*. A total of 24 rats were randomly divided into two groups, each receiving a tail vein injection, as indicated: Adriamycin (ADR) (7 mg/kg in saline); control (equal volume of saline). After 2 weeks, half of each group ($n = 6$) received the sEH inhibitor AUDA by oral gavage at a dose of 0.5 mg/kg per day [16,17]. After 4 weeks of AUDA treatment all animals were sacrificed and kidneys were harvested.

2.4. Quantitative real-time reverse transcriptase PCR (qRT-PCR)

Transcript levels of sEH, E-cadherin and α-SMA were analyzed using quantitative real-time reverse transcriptase PCR (qRT-PCR). Briefly, total RNA was isolated from cultured cells or renal cortex with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and concentrations assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then, 1 μg of total RNA was reverse-transcribed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed using SYBR green Master Mix reagent (Promega) with a Bio-Rad real-time PCR machine according to the manufacturer's instructions. The relative quantification of gene expression was calculated as $2^{-\Delta\Delta Ct}$. The primer sequences used were: sEH-forward, 5′-AAGCCTGTGGAGCCAGTCTA-3′, reverse, 5′- CCAGTTGTTGGTGACAAT GC-3′; E-cadherin-forward, 5′-AACGAGGGCATTCTGAAAACA-3′, reverse, 5′-CACTGTCAC GTGCAGAATGTACTG-3′; α-SMA-forward, 5′-GACCCTGAAGTATCCGATAGAACA-3′, reverse, 5′- CACGCGAAGCTCGTTATAGAAG-3′; β-Actin-forward, 5′-AGAGCTATGAGCTG CCTGAC-3′, reverse, 5′-AATTGAATG TAGTTTCATGGATG-3′.

2.5. Western blotting

Total protein was isolated from cultured cells or kidney cortex and quantified using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Samples (45 μg protein per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a nitrocellulose membrane, proteins were incubated with the following primary antibodies overnight at 4 °C: anti-sEH (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-E-cadherin (1:200, Santa Cruz Biotechnology); anti-α-SMA (1:1000, Abcam, San Diego, CA, USA); anti-PI3K (1:1000; CST, Danvers, MA, USA); anti-Akt (1:2000; CST); anti-p-Akt (Ser 473; 1:2000; CST); anti-GSK-3β (1:1000; CST); anti-p-GSK-3β (Ser 9; 1:1000; CST). Fluorescently labeled secondary antibodies (1:10000; LI-COR Biosciences, Lincoln, NE, USA) were used to detect the binding of the primary antibodies. The bound proteins were visualized by scanning the membranes in an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.6. Statistical analysis

Data were expressed as the means ± standard deviation and analyzed using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). For comparisons of multiple groups, a one way analysis of variance (ANOVA) followed by a least square difference (LSD) multiple comparison test was performed. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. sEH participated in UP-induced EMT

Exposure to UP for 48 h resulted in morphological conversion of NRK-52E cells from an epithelial to a myofibroblast-like phenotype (Fig. 1A), with decreased expression of the characteristic epithelial adhesion molecule E-cadherin and increased expression of the myofibroblast-specific marker α-SMA (Fig. 1B, C and D). In particular, expression of sEH was increased at both the mRNA and protein levels. Treatment with sEH inhibitor AUDA blunted this phenotypic transition and significantly attenuated the decrease in E-cadherin and the increase in α-SMA, suggesting that sEH played an important role in the UP-induced EMT. Consistent with previous studies [18], sEH expression had no significant change with the treatment

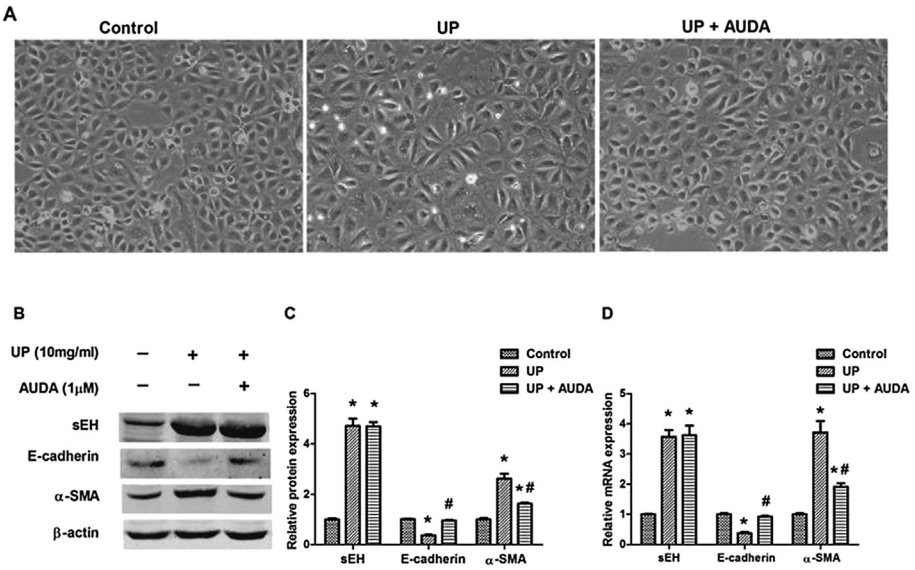

Fig. 1. Upregulation of sEH mediated UP induced EMT in NRK-52E cells. NRK-52E cells were incubated with UP (10 mg/ml) for 48 h in the presence or absence of sEH inhibitor AUDA (1 μM) (n = 3). (A) UP exposure induced morphological (epithelial to fibroblastic) changes in NRK-52E cells, which was partially reversed by AUDA treatment (Magnification, × 100). (B) Representative Western blotting images of sEH, E-cadherin and α-SMA. (C and D) Treatment with AUDA significantly attenuated UP induced E-cadherin downregulation and α-SMA upregulation at both the protein and mRNA levels. $^{*}P < 0.05$ vs. Control group; $^{\#}P < 0.05$ vs. UP group.

of AUDA, indicating that AUDA is effective at inhibiting sEH catalytic activity without altering its expression.

3.2. PI3K-Akt-GSK-3β signaling pathway was involved in UP-induced EMT

To examine the mechanism of the UP-induced EMT process, we measured activity of the PI3K-Akt-GSK-3β pathway in NRK-52E cells after UP incubation for different periods of time. UP caused a rapid activation of Akt (Fig. 2A) and augmented phosphorylation of GSK-3β (Fig. 2B) in the cells. As expected, pretreatment of cells with a PI3K inhibitor (LY-294002, 25 μM) reduced UP-induced phosphorylation of both Akt and GSK-3β (Fig. 2C and D). More importantly, LY-294002 significantly attenuated the EMT associated decrease in E-cadherin and increase in α-SMA (Fig. 2E, F and G) as well as the phenotypic cellular changes (Fig. 2H) without altering sEH expression.

3.3. sEH inhibitor regulated PI3K-Akt-GSK-3β pathway in UP-induced EMT

As shown in Fig. 3, pretreatment with the sEH inhibitor AUDA partially suppressed the UP-induced increase in PI3K activation and phosphorylation of Akt and GSK-3β. Considering UP-induced EMT was partially reversed by AUDA (Fig. 1), these data suggest that the suppression of UP-induced EMT by sEH inhibition involves the PI3K-Akt-GSK-3β signaling pathway.

3.4. sEH inhibitor ameliorated renal EMT by regulating the PI3K-Akt-GSK-3β pathway in rats with ADR-induced nephropathy

To explore the effects of sEH on EMT *in vivo*, we employed a rat model for ADR-induced nephropathy. Western blotting and qRT-PCR analyses showed that, compared with that from controls, renal tissue from rats treated with ADR showed significant downregulation of E-cadherin and upregulation of α-SMA. These changes were associated with increased sEH expression, PI3K activation and increased Akt and GSK-3β phosphorylation. In contrast, treatment with sEH inhibitor for 4 weeks markedly reduced renal PI3K expression and restrained the phosphorylation of Akt and GSK-3β (Fig. 4A and B), as compared with those from the ADR group. Additionally, E-cadherin decrease and α-SMA increase were also attenuated (Fig. 4C, D and E). Collectively, these data indicate that the suppressive effect of sEH inhibitor on renal EMT is related to the PI3K-Akt-GSK-3β pathway in rats with chronic proteinuric renal disease.

4. Discussion

The major focus of the present study was to determine the role of sEH in proteinuria-induced EMT and to elucidate its underlying mechanism of action. We demonstrated that a 48 h exposure to UP-induced EMT and elevated sEH expression in renal proximal tubular epithelial cells. Importantly, the PI3K-Akt pathway was activated while GSK-3β was inactivated, as evidenced by marked upregulation of PI3K, p-Akt and p-GSK-3β after UP treatment. Pharmacological inhibition of sEH suppressed PI3K-Akt activation, ablated GSK-3β phosphorylation and blocked UP-induced EMT. *In vivo*, sEH inhibition ameliorated renal EMT by regulating PI3K-Akt-GSK-3β activation in rats with ADR-induced nephropathy.

It has been reported that a single component of UP, such as albumin or complement, can trigger EMT [4,5]. However, such studies with isolated proteins do not comprehensively explore the possible effects of the mixture of various proteins present in urine. In our study, UP was extracted from patients with minimal change disease, one of the most common primary glomerulonephritis observed clinically. Compared with the isolated components, this UP preparation could better mimic the clinical situation. It has been suggested that UP increases tubular synthesis of transforming growth factor-β (TGF-β), which is a potent stimulus for EMT [19]. In the present study, UP exposure for 48 h induced significant downregulation of E-cadherin and upregulation of α-SMA in cultured proximal tubular epithelial cells, highlighting proteinuria as a key regulator of EMT.

Notably, we found that sEH expression was markedly elevated in UP-induced EMT. AUDA, a potent inhibitor of sEH, attenuated the increase of α-SMA and normalized the E-cadherin expression in the presence of UP. sEH has been identified as a major determinant of

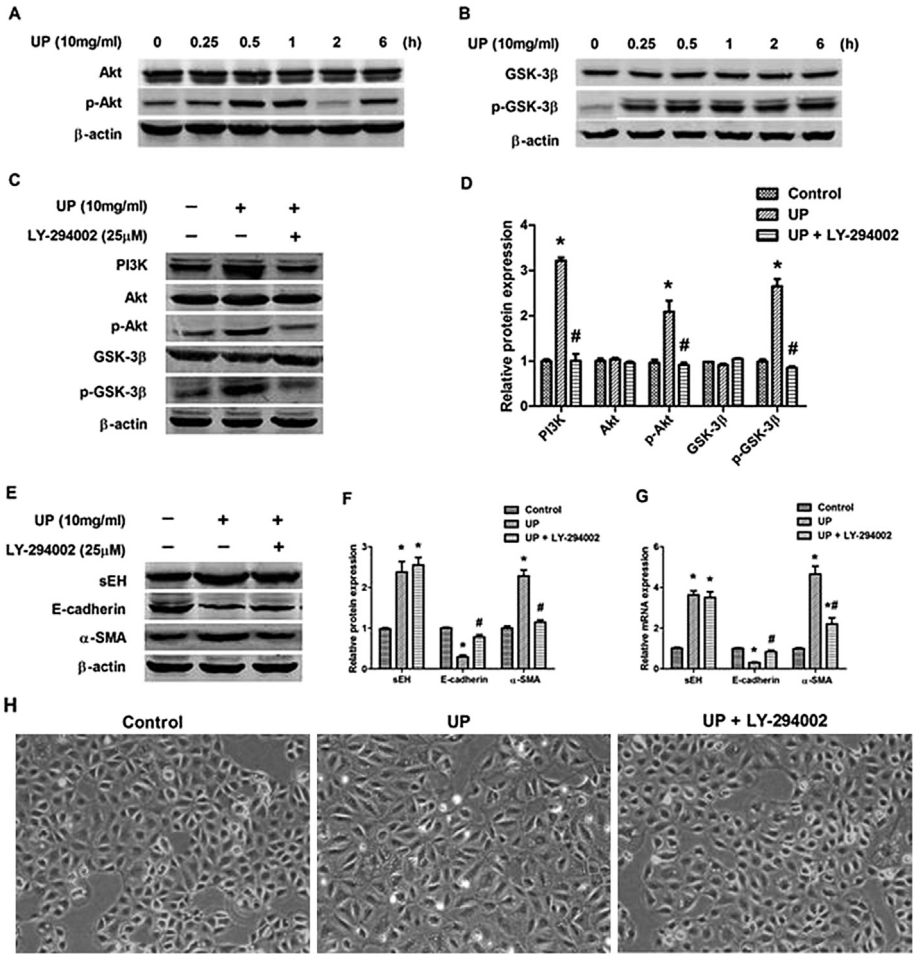

Fig. 2. The PI3K-Akt-GSK-3β signaling pathway was involved in UP induced EMT. NRK-52E cells were incubated with UP (10 mg/ml) in the presence or absence of PI3K inhibitor LY-294002 (25 μM) (n = 3). (A and B) Phosphorylation of Akt and GSK-3β induced by UP for the period of time indicated. (C and D) NRK-52E cells were incubated with LY-294002 (25 μM) for 30 min before the UP incubation for 30 min. LY-294002 inhibited UP induced PI3K-Akt activation and GSK-3β phosphorylation. (E, F and G) NRK-52E cells were incubated with LY-294002 (25 μM) for 30 min before the UP incubation for 48 h. LY-294002 pretreatment attenuated EMT associated E-cadherin reduction and α-SMA increase without altering sEH expression. (H) LY-294002 blunted the phenotypic transition induced by UP in NRK-52E cells (Magnification, × 100). $^{*}P < 0.05$ vs. Control group; $^{\#}P < 0.05$ vs. UP group.

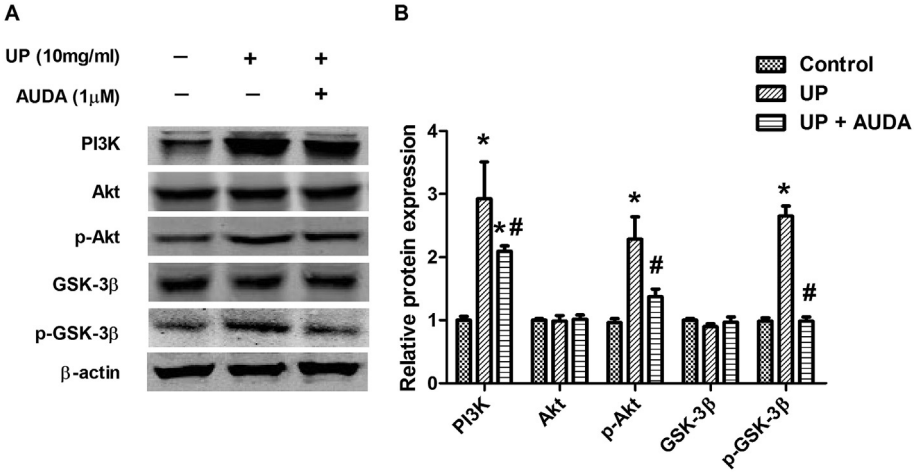

Fig. 3. AUDA regulated PI3K-Akt-GSK-3β pathway in UP induced EMT. NRK-52E cells were incubated with sEH inhibitor AUDA (1 μM) for 30 min before the UP (10 mg/ml) incubation for 30 min (n = 3). (A) Representative Western blotting images. (B) AUDA markedly reduced PI3K activation and phosphorylation of Akt and GSK-3β. $^{*}P < 0.05$ vs. Control group; $^{\#}P < 0.05$ vs. UP group.

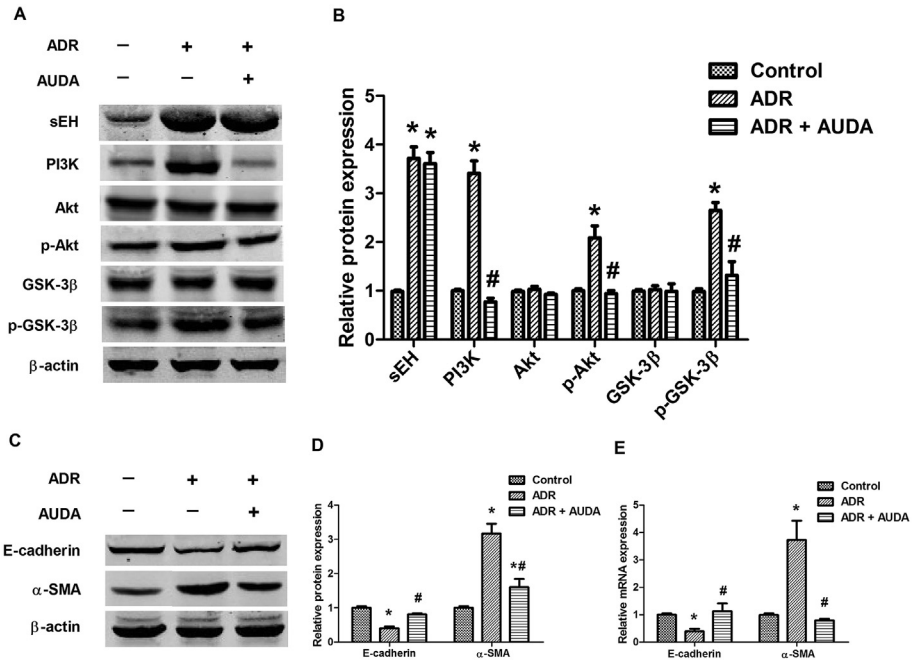

Fig. 4. AUDA ameliorated renal EMT by regulating PI3K-Akt-GSK-3β signaling pathway in rats with ADR-induced nephropathy. Rats with ADR-induced nephropathy were treated with AUDA at a dose of 0.5 mg/kg per day for 4 weeks (n = 6). (A and B) AUDA inhibited renal PI3K-Akt signaling activation and GSK-3β phosphorylation in rats with ADR-induced nephropathy. (C, D and E) Renal E-cadherin downregulation and α-SMA upregulation were attenuated by AUDA treatment. $^{*}P < 0.05$ vs. Control group; $^{\#}P < 0.05$ vs. ADR group.

the bioavailability of EETs. In previous reports, sEH was described as contributing to renal tubular damage in diabetic nephropathy and hypertension through its hydrolysis of EETs [9,10]. Additionally, synthetic EETs attenuated TGF-β1-induced EMT in cultured human proximal tubular epithelial cells [20]. Taken together, the evidence indicates that upregulation of sEH mediates proteinuria-induced EMT by decreasing the protective effects of EETs on renal tubular epithelial cells.

Many intracellular signaling molecules have been implicated in the pathogenesis of EMT. The PI3K-Akt pathway is activated in tubular epithelial EMT induced by hypoxia or high glucose [12,13]. GSK-3β, a downstream target of PI3K-Akt signaling, is also necessary for maintenance of epithelial architecture. Phosphorylation and functional inactivation of GSK-3β is important for Snail expression and β-catenin nucleus translocation, both typical molecular changes during EMT [21,22]. In agreement with previous studies, we found that the PI3K-Akt pathway was activated in UP-induced EMT, an effect correlated with hyperphosphorylated GSK-3β. Pharmacological inhibition of PI3K with LY-294002 reduced Akt (Ser 473) and GSK-3β (Ser 9) phosphorylation, thus attenuating changes in expression of epithelial and mesenchymal markers. These data suggest that the PI3K-Akt-GSK-3β signaling pathway plays a critical role in UP-induced EMT. More important, in the presence of UP, sEH inhibition by AUDA significantly downregulated PI3K expression and reduced phosphorylation of both Akt and GSK-3β. Therefore, it is plausible to assume that the effect of sEH inhibition on UP-induced EMT is mediated by inactivation of PI3K-Akt and hypophosphorylation of GSK-3β. However, it should be noted that, in a study with diabetic mice, PI3K and Akt phosphorylation levels were significantly decreased in the kidneys and sEH gene disruption partially reversed these effects [10]. The precise mechanisms underlying the sEH regulation of PI3K/Akt, therefore, remain unclear. We interpret with caution that different treatment options and disease states might be responsible for the discrepancy. Li and colleagues [23] demonstrated that, in a model for Angiotensin II-induced cardiac dysfunction, partial sEH disruption by pharmacological inhibition had beneficial effects while, in contrast, total sEH deletion shifted arachidonic acid metabolism and therefore had deleterious effects. These findings support our hypothesis because our study used pharmacological sEH inhibition rather than complete gene disruption.

ADR-induced nephropathy is a commonly used model of chronic proteinuric renal disease. Because of the cytotoxic effects of ADR on podocytes, rats develop overt proteinuria and subsequent renal tubular injury [24]. In the present study, we found that E-cadherin was decreased and α-SMA increased in the renal cortex from rats with ADR-induced nephropathy, indicating that EMT had been induced in the kidneys. As either tubular epithelial cells or podocytes can undergo EMT upon exposure to proteinuria [25,26], it is difficult to determine which cell is responsible for the observed changes in E-cadherin and α-SMA in kidney tissue. We believe that both cells are involved. Importantly, treatment with a sEH inhibitor attenuated the abnormal expression of EMT markers induced by proteinuria. Additionally, PI3K-Akt overactivation and GSK-3β hyperphosphorylation were suppressed. These findings confirm our observations *in vitro*. Tubular epithelial EMT is an important process leading to renal tubulointerstitial fibrosis. The effects of modulating sEH in EMT support its critical role in renal fibrosis. As further support, recent studies demonstrated that sEH disruption prevented renal interstitial fibrogenesis in experimental obstructive nephropathy [27–29].

In conclusion, we demonstrated for the first time that sEH inhibition prevents proteinuria-induced EMT in renal proximal tubular epithelial cells via the PI3K-Akt-GSK-3β signaling pathway. Our findings suggest that targeting sEH might be a promising therapeutic strategy to treat EMT and renal fibrosis.

Conflict of interest

None.

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Transparency document

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