LABORATORY STUDY

Metformin alleviated EMT and fibrosis after renal ischemia–reperfusion injury in rats

Min Wang, Xiaodong Weng, Jia Guo, Zhiyuan Chen, Guanjun Jiang and Xiuheng Liu

Department of Urology, Renmin Hospital of Wuhan University, Wuhan University, Wuhan, China

ABSTRACT

Purpose The purpose of this study is to assess the potential effects of metformin on the development of EMT and tubulointerstitial fibrosis 12 weeks after acute renal ischemia–reperfusion.

Methods Male Sprague–Dawley rats were randomly assigned to four groups: Sham, IRI, transient administration of metformin (TAM), and continuous administration of metformin (CAM). Metformin was administered i.p. at a dose of $125 \mu g kg^{-1} d^{-1}$ 3 d prior to suffering from IRI (TAM), or from 3 d before suffering from IRI to 12 weeks after reperfusion (CAM). Renal function, histology, and expressions of IL-6, TNF-$\alpha$, $\alpha$-SMA, TGF-$\beta$1, Vimentin, and E-cadherin were analyzed.

Results Tubulointerstitial fibrosis worsened further in IRI, accompanied by the increased expressions of interleukin-6, TNF-$\alpha$, $\alpha$-SMA, TGF-$\beta$1, Vimentin, and loss of E-cadherin. Although there were no significant differences between IRI and TAM ($p > 0.05$). Compared with the IRI, expressions of IL-6, TNF-$\alpha$, $\alpha$-SMA, TGF-$\beta$1, and Vimentin were reduced and the expression of E-cadherin was restored in CAM ($p < 0.05$). CAM also significantly promoted activation of AMPK ($p < 0.05$), which showed no difference among Sham, IRI, and TAM ($p > 0.05$). Conclusions CAM significantly attenuated tubulointerstitial fibrosis and EMT in rats, potentially via activation of AMPK and down-regulation of TGF-$\beta$1.

Introduction

Despite many advances in surgery, the incidence of ischemia–reperfusion injury (IRI) was still inevitable for patients who received kidney transplant and major aortic surgery. With the exception of many factors, including inflammatory, endothelial, toxic and metabolic, ischemia was a very important risk factor which might result in renal fibrosis and finally chronic kidney disease (CKD).1

Renal fibrosis was considered as a maladaptive repair process characterized by glomerulosclerosis, tubular atrophy, interstitial fibrosis, and capillary rarefaction. Similar to wound healing, renal fibrosis initiated a probably beneficial response to the injured kidney.2 However, according to the duration, type, and severity of injury, this beneficial response could be subsequently progressed to a phase called fibroplasia or fibrosis, in which functional tissue was remodeled and gradually replaced by connective tissue, leading to irreversible scar formation.3

Epithelial–mesenchymal transition (EMT) played a critical role in a series of process, such as embryonic development, wound healing, tissue regeneration, cancer progression, as well as organ fibrosis. In the past several years, EMT had emerged as one of the most interesting topics in the field of renal fibrosis, and had caused widespread concern.4–6 In the kidney, TGF-$\beta$1 had been widely regarded as the primary mediator of renal fibrosis and EMT.7–9 Moreover, the progression of fibrosis was associated with chronic inflammation and characterized by increased extracellular matrix (ECM) synthesis, such as increased expression of $\alpha$-SMA, Vimentin, and loss of E-cadherin.

Metformin was an orally administered biguanide drug with an insulin-sensitizing effect, which was widely used in patients with diabetes mellitus. The AMP-activated protein kinase (AMPK) played a key role in the regulation of metabolism, and its activation resulted in the down-regulation of several anabolic processes, such as fatty acid oxidation, glucose uptake, and glycolysis. Metformin was the best known activator of AMPK and was found to protect heart and kidney from IRI.10–12 Metformin was also effective on against TGF-$\beta$1-induced EMT in cancer and kidney fibrosis.13 Moreover,
metformin could also attenuate the process of remodeling and fibrosis of heart after IRI, potentially via reducing expression of TGF-β1 and collagen expression. Meanwhile, metformin reduces renal remodeling in an animal model of diabetic nephropathy and ameliorates liver fibrosis in clinical.

Then, we speculated that metformin might also ameliorate renal fibrosis by inhibiting chronic inflammation and process of EMT. However, there was no study which discussed protective effect of metformin on renal fibrosis after IRI. In the present study, we investigated the role of metformin in ischemia-induced renal fibrosis. Moreover, the related molecules IL-6, TNF-α, α-SMA, TGF-β1, Vimentin, E-cadherin, and AMPK were tested to explore whether and how metformin affected the renal EMT and renal fibrosis.

Methods and material

Animal preparation

Adult male Sprague–Dawley rats (250–280 g) were obtained from the experimental animal center of Union Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology. The committee for experimental animals of Wuhan University approved all experimental procedures, and the procedures complied with the Guidelines for the Care and Use of Laboratory Animals. Rats were anesthetized with pentobarbital (45 mg/kg). In brief, the midline laparotomy was performed and, after right nephrectomy, the left kidney was subjected to 45 min of ischemia followed by reperfusion. During the procedure, core body temperatures were maintained at 37°C on a homeothermic table.

Animals were randomly divided into four groups (five rats per group, n = 5): Sham group, IRI group and IRI + metformin group (including transient administration of metformin (TAM) group and continuous administration of metformin (CAM) group). In the sham group, the kidneys were treated identically with the IRI group, without clamping the left pedicle; in the ischemic reperfusion injury (IRI) group, kidneys were subjected to 45 min of ischemia followed by reperfusion; in the TAM group, metformin was administered i.p at a dose of 125 μg kg⁻¹ d⁻¹ 3 d prior to suffering from IRI; in the CAM group, metformin was administered i.p at a dose of 125 μg kg⁻¹ d⁻¹ from 3 d before suffering from IRI to 12 weeks after reperfusion.

The rats were then killed at the end of 12th week after reperfusion. Blood was obtained via puncture of the inferior vena cava, and the left kidney was removed under fully maintained anesthesia. After removal, the kidneys were fixed in 10% phosphate-buffered formalin or immediately frozen, and stored at −80°C for different procedures.

Renal function analysis

Serum Cr and blood urea nitrogen (BUN) were determined, using standard techniques and an Olympus AU2700 Analyzer (Olympus, Optical Co., Tokyo, Japan).

Histological examinations

For histological preparations, kidneys were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned into 5 μm thick sections according to the standard procedure. The sections were deparaffinized and hydrated gradually, and stained with hematoxylin and eosin (H&E) and Masson’s trichrome. Morphological assessments were performed blindly by an experienced renal pathologist. Long-term tubulointerstitial injury was evaluated based on calculating the percentage of the affected area fraction as described previously, 10 fields per section at ×400 magnification.

Immunohistochemistry

The expression of TGF-β1 was investigated. Tissue sections were stained by immunohistochemistry (IHC) using specific antibodies for TGF-β1 (mouse monoclonal, 1:500, Cell Signal Technology, Boston, MA). Serial sections (thickness 5 μm) were cut from the tissue blocks, deparaffinized in xylene, and hydrated in a graded series of alcohol. Staining was then performed using the DAB chromogenic agent (Dako Corp, Carpinteria, CA). Negative control experiments were routinely performed. All slides were evaluated by an experienced renal pathologist who was unaware of the origin of the slides.

Western blot analysis

The protein expression levels of IL-6, TNF-α, α-SMA, TGF-β1, Vimentin, and E-cadherin were examined by Western blotting. Briefly, proteins were extracted from kidneys, separated on 10–12% SDS-PAGE gels (40 μg/lane) and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk in TBST buffer (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.05% Tween 20, pH 7.2) for 2 h and incubated with primary antibodies overnight at 4°C. Primary antibodies used here were monoclonal mouse antibodies against IL-6 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), α-SMA (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and polyclonal rabbit
antibodies against TNF-α (1:1000 dilution; Cell Signaling Technology, Boston, MA), TGF-β1 (1:1000 dilution; Cell Signaling Technology, Boston, MA), Vimentin (1:1000 dilution; Cell Signaling Technology, Boston, MA), E-cadherin (1:1000 dilution; Cell Signaling Technology, Boston, MA), and AMPK (1:1000 dilution; Cell Signaling Technology, Boston, MA). After extensive washing with TBST buffer, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were detected using an enhanced chemiluminescence system (ECL kit, Pierce Biotechnology, Beijing, China) and captured on lightsensitive X-ray film (Kodak, Shanghai, China). Optical densities were detected using ImageJ software.

Real-time PCR (RT-PCR)

For gene re-expression studies RT-PCR was performed. Total RNA were isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and RNA concentration was obtained by spectrophotometer. Single-stranded cDNA was synthesized using the cDNA synthesis kit (Takara, Kyoto, Japan) according to the procedures. The primers used were as follows: α-SMA (Forward: GCTCCTCCAGAACGAAATAT, Reverse: GGGCCAGCTTCGTCATACTC); E-cadherin (Forward: ATGAGGTCGGTGCGTTATT, Reverse: CGTTGGTCCTGCTGGA); GAPDH (Forward: CCGTGACGAGCTGGTTA, Reverse: CCATCACGCCACAGCTTTCC). A total of 40 cycles of amplification was performed for each of the RT-PCR experiments. GAPDH was amplified as an internal control. Amplified products were analyzed on 1.5–3% agarose gels with Goldview staining (Beijing Baihao Biological Technology Company, Beijing, China).

Statistical analysis

All data were presented as mean ± SEM. Differences were considered statistically significant when p values were <0.05. The means of the different groups were compared using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test. The Kruskal–Wallis ANOVA on ranks was used for non-normally distributed data.

Results

Renal function

BUN and Cr levels were measured at 12 weeks following IRI in rats. In this model, renal function was not altered significantly and the treatment with metformin did not change these results (Figure 1).

Morphological features and immunohistochemistry

Morphological features were evaluated by using H&E (Figure 2A–D) and Masson’s trichrome staining (Figure 2E–H). From the serial observation, it indicated that glomeruli sizes tended to be obviously reduced in the IRI group and the TAM group, in which it was common to see the characterization of patchy tubulointerstitial injury and fibrosis, such as infiltration of massive inflammatory cells accompanying tubular injury, tubular dilation or atrophy, and interstitial fibrosis. Compared with the sham group, the IRI, the TAM and the CAM group showed significant increases in the sizes of tubulointerstitial fibrotic area, but renal tubulointerstitial fibrotic areas were comparatively attenuated in the CAM group (Figure 2M).

In our study, TGF-β1 was detected by immunohistochemistry staining (Figure 2I–L). It revealed that TGF-β1 was rarely found in the sham group. But in the IRI group, renal tissues were strongly positive for TGF-β1 expression, mainly in the injured renal tubular epithelial cell and tubulointerstitium. Compared with the IRI group,
these expressions were ameliorated in the CAM group, but were not ameliorated in the TAM group (Figure 2N).

**Western blot**

The results of Western blot indicated that the expressions of IL-6, TNF-α, α-SMA, TGF-β1, and Vimentin were upregulated in the IRI group and the IRI+Metformin group when compared with the sham group (Figure 3). But treatment with continuous administration of metformin could attenuate these expression induced by IRI, but transient administration of metformin could not. Compared with sham group, E-cadherin was down regulated in rats subjected to IRI. However, the expression of E-cadherin in the CAM group was obviously more than that observed in the IRI group, but the expression of E-cadherin are not different between the TAM group and the IRI group (Figure 3). In addition, the activation of AMPK was not different among the TAM group, the IRI group, and the sham group. However, continuous administration of metformin significantly activated AMPK (Figure 4).

**RT-PCR analysis**

To investigate the mRNA expression of α-SMA and E-cadherin, we measured their levels by RT-PCR. The relative expression of α-SMA and E-cadherin to GAPDH was shown (Figure 5). The mRNA levels of α-SMA was significantly greater in the CAM group than in the sham group, but was significantly less than the IRI group and the TAM group. Moreover, the expressions
Discussion

The present study demonstrated that metformin could effectively attenuate renal fibrosis following IRI in rats. The results suggested that metformin might attenuate renal fibrosis by inhibiting chronic inflammation and process of EMT via AMPK activation.

This study suggested that continuous administration of metformin might attenuate renal fibrosis by decreasing the expression of TNF-α and IL-6, both of which had been consistently implicated in the pathogenesis of renal fibrosis. Renal fibrosis was a chronic progressive process, which could result from chronic inflammation caused by IRI. TNF-α could directly stimulate TGF-β1 production, collagen deposition in the renal cortex, increased interstitial volume, and deteriorating renal fibrosis,\(^18,19\) TNF-α might also play an important role in EMT, perhaps by modulating TGF-β1 activity.\(^20,21\) In our
study, continuous administration of metformin successfully reduced the expression of TNF-α and IL-6, but transient administration of metformin did not.

Renal fibrosis was characterized by excessive accumulation of ECM proteins, including increased expression of α-SMA, Vimentin, and loss of E-cadherin. Fibrosis led to gradual expansion of the fibrotic mass which destroyed the normal tissue and ultimately resulted in ESRD. The pathophysiology of renal fibrosis was still uncertain, but significant evidence revealed that EMT was an important component of this process. Emerging evidence indicated that mature tubular epithelial cells were capable of transforming into myofibroblasts under pathological conditions, a process that was called EMT. TGF-β1 had been described as a major driving force of EMT of renal tubules and renal fibrosis. This cytokine was synthesized from various cell lines, such as renal tubular epithelial cells,
macrophages and interstitial fibroblasts. Over-expression of TGF-β1 in renal tubular epithelial cells induced comprehensive peritubular proliferation of resident fibroblasts, differentiation into myofibroblasts, and subsequent progressive deposition of ECM.

TGF-β1-induced EMT appeared to mainly depend on the intact Smad signaling. Activation of TGF-β1 caused phosphorylation of Smad-2 and 3 by its type I receptor, which also induced their association with Smad-4. This complex was then translocated into the nucleus to dominate the transcription of TGF-β1 responsive genes.

This study suggested that continuous administration of metformin might relieve EMT and renal fibrosis via diminishing TGF-β1 expression. As we know, TGF-β1 played a pivotal role in the process of EMT and fibrosis, which were characterized by the increased expression of α-SMA, Vimentin, and loss of E-cadherin. In this study, continuous administration of metformin significantly reduced the expression of TGF-β1, as well as reduced the expression of α-SMA and Vimentin, and effectively increased E-cadherin expression.

In addition, this study also suggested that continuous administration of metformin might alleviate EMT and renal fibrosis via activation of AMPK. Recently, several studies had suggested that activation of AMPK exhibited anti-inflammatory and anti-tissue remodeling functions. And metformin was the best-known clinical activator of AMPK. Our results showed that continuous administration of metformin significantly activated AMPK. On the contrary, activation level of AMPK was not different among the TAM group, the IRI group and the sham group.

There were some limitations in this study. First, metformin was an oral anti-hyperglycemic agent widely used for the treatment of type 2 diabetes. But, it was administration by intraperitoneal injection in this study. Second, the dosage used in this study was less than conventional dosage. Finally, this study did not discuss the effect of AICAR (an activator of AMPK) and compound C (an inhibitor of AMPK). So, it was uncertain whether metformin alleviated EMT and renal fibrosis via activation of AMPK or not. So, further studies were needed to validate these issues.

There was no effective therapy to cure renal fibrosis and ESRD. Restoration of modulating molecular targets, such as TGF-β1, α-SMA, Vimentin, and E-cadherin, involved in EMT might be a new way to halt or even reverse renal fibrosis. Metformin, a well-known activator of AMPK, could significantly attenuate the process of EMT and renal fibrosis following IRI in rat. Metformin was a cheap, safe, and widely used drug. It appeared that metformin might offer a valuable potential strategy to protect against ischemic-induced fibrosis.

Acknowledgements

The authors thank for Key Laboratory of Hubei Province for Digestive System Disease and for all the authors whose work was included in this study.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding information

This study is supported by the grants from the National Natural Science Foundation of China (No.2013RMFH012), the Province Natural Science Foundation of Hubei (No. 2012FFA096), and supported by the Fundamental Research Funds for the Central Universities (No. 2042014kf0115).

References

1. Schlondorff DO. Overview of factors contributing to the pathophysiology of progressive renal disease. Kidney Int. 2008;74:860–866.
2. Lin SL, Li B, Rao S, et al. Macrophage Wnt7b is critical for kidney repair and regeneration. Proc Natl Acad Sci USA. 2010;107:4194–4199.
3. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008;214:199–210.
4. Guarino M, Tosoni A, Nebuloni M. Direct contribution of epithelium to organ fibrosis: Epithelial–mesenchymal transition. Hum Pathol. 2009;40:1365–1376.
5. Jiang T, Huang B, Chen PS, et al. Epithelial–mesenchymal transition of renal tubules: Divergent processes of repairing in acute or chronic injury? Med Hypotheses. 2013;81:73–75.
6. Burns WC, Kantharidis P, Thomas MC. The role of tubular epithelial-mesenchymal transition in progressive kidney disease. Cells Tissues Organs. 2007;185:222–231.
7. Wang Q, Usinger W, Nichols B, et al. Cooperative interaction of CTGF and TGF-β1 in animal models of fibrotic disease. Fibrogenesis Tissue Repair. 2011;4:4.
8. Yeh YC, Wei WC, Wang YK, et al. Transforming growth factor-β1 induces Smad3-dependent (β1) integrin gene expression in epithelial-to-mesenchymal transition during chronic tubulointerstitial fibrosis. Am J Pathol. 2010;177:1743–1754.
9. Zeng R, Han M, Luo Y, et al. Role of Sema4C in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial–mesenchymal transition in renal tubular epithelial cells. Nephrol Dial Transplant. 2011;26:1149–1156.
10. El Messaoudi S, Rongen GA, de Boer RA, et al. The cardioprotective effects of metformin. Curr Opin Lipidol. 2011;22:445–453.
11. Lin A, Sekhon C, Sekhon B, et al. Attenuation of ischemia–reperfusion injury in a canine model of autologous renal transplantation. Transplantation. 2004;78:654–659.
12. Seo-Mayer PW, Thulin G, Zhang L, et al. Preactivation of AMPK by metformin may ameliorate the epithelial cell damage caused by renal ischemia. Am J Physiol Renal Physiol. 2011;301:F1346–F1357.
13. Cufi S, Vazquez-Martin A, Oliveras-Ferraros C, et al. Metformin against TGFβ-induced epithelial-to-mesenchymal transition (EMT): From cancer stem cells to aging-associated fibrosis. Cell Cycle. 2010;9:4461–4468.

14. Xiao H, Ma X, Feng W, et al. Metformin attenuates cardiac fibrosis by inhibiting the TGFbeta1-Smad3 signalling pathway. Cardiovasc Res. 2010;87:504–513.

15. Lee MJ, Felliars D, Mariappan MM, et al. A role for AMP-activated protein kinase in diabetes-induced renal hypertrophy. Am J Physiol Renal Physiol. 2007;292:F617–F627.

16. de Oliveira CP, Stefano JT, de Siqueira ER, et al. Combination of N-acetylcysteine and metformin improves histological steatosis and fibrosis in patients with non-alcoholic steatohepatitis. Hepatol Res. 2008;38:159–165.

17. Nankivell BJ, Borrows RJ, Fung CL, et al. The natural history of chronic allograft nephropathy. N Engl J Med. 2003;349:2326–2333.

18. Guo G, Morrissey J, McCracken R, et al. Role of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy. Am J Physiol. 1999;277:F766–F772.

19. Meldrum KK, Misseri R, Metcalfe P, et al. TNF-alpha neutralization ameliorates obstruction-induced renal fibrosis and dysfunction. Am J Physiol Regul Integr Comp Physiol. 2007;292:R1456–R1464.

20. Dong R, Wang Q, He XL, et al. Role of nuclear factor kappa B and reactive oxygen species in the tumor necrosis factor-alpha-induced epithelial–mesenchymal transition of MCF-7 cells. Braz J Med Biol Res. 2007;40:1071–1078.

21. Chaudhuri V, Zhou L, Karasek M. Inflammatory cytokines induce the transformation of human dermal microvascular endothelial cells into myofibroblasts: A potential role in skin fibrogenesis. J Cutan Pathol. 2007;34:146–153.

22. Sato M, Muragaki Y, Saika S, et al. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J Clin Invest. 2003;112:1486–1494.

23. Misseri R, Rink RC, Meldrum DR, et al. Inflammatory mediators and growth factors in obstructive renal injury. J Surg Res. 2004;119:149–159.

24. Pairs A, Radjabi A, Davis S, et al. Activation of AMPK inhibits inflammation in MRL/Lpr mouse mesangial cells. Clin Exp Immunol. 2009;156:542–551.

25. Lee JH, Kim JH, Kim JS, et al. AMP-activated protein kinase inhibits TGF-β1, angiotensin II, aldosterone, high glucose-, and albumin-induced epithelial-mesenchymal transition. Am J Physiol Renal Physiol. 2013;304:F686–F697.