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Metformin prevents renal interstitial fibrosis in mice with unilateral ureteral obstruction

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ABSTRACT

Unilateral ureteral obstruction causes important tubulo-interstitial fibrosis in the kidney. Metformin reduces fibrosis in mice with diabetic nephropathy. We examined the effects of metformin in a mouse model of unilateral ureteral obstruction (UUO). Expression of inflammation and fibrosis markers was studied by immunohistochemistry, immunoblot and quantitative real-time polymerase chain reaction. Seven days after UUO, kidneys presented dilated tubules, expansion of the tubulo-interstitial compartment, and significant infiltration of inflammatory cells. Macrophage infiltration and inflammation markers expression were increased in obstructed kidneys and reduced by metformin. Metformin reduced expression of extracellular matrix proteins and profibrotic factor TGFβ in obstructed kidneys, measured by immunohistochemistry. Interstitial fibroblast activation was evident in obstructed kidneys and ameliorated by metformin. UUO did not affect adenosine monophosphate-activated kinase (AMPK) activity, but metformin activated AMPK. Our results show that metformin prevents or slows down the onset of renal inflammation and fibrosis in mice with UUO, an effect that could be mediated by activation of AMPK.

1. Introduction

Obstructive nephropathy can occur in both children and adults. In adults, renal function usually recovers after elimination of the obstruction, but in children obstruction can have long term consequences. Fetal and neonatal obstruction result in reduced nephron number, with the magnitude of reduction being dependent on the severity and duration of obstruction (Chevalier et al., 2009). Obstruction is accompanied by changes in kidney structure, inflammation and tubulointerstitial fibrosis, leading to decrease and eventually loss of renal function if the obstruction persists. Interstitial fibrosis is a major prognostic indicator of end-stage renal disease (Eddy, 2000). Inhibition of interstitial fibrosis is therefore critical to preserve kidney function (Eddy, 2005). Unilateral ureteral obstruction (UUO) is an experimental model for tubulo-interstitial fibrosis (Chevalier et al., 2009). UUO causes renal hemodynamic and metabolic changes, leading to tubular injury and renal inflammation, characterized by macrophage infiltration. Activation of interstitial fibroblasts causes their differentiation into myofibroblasts, which contribute to accumulation of the extracellular matrix proteins, such as fibronection, collagens I and II and lead eventually to renal fibrosis (for a review, see Chevalier et al., 2009). Upon recruitment and activation, macrophages produce various pro-inflammatory cytokines, such as TNFα and TGFβ which in turn promote expression of adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM1), and contribute to further recruitment of circulating inflammatory cells (see Chevalier et al., 2010 for a review).

Metformin is a biguanide compound that has been used as an oral anti diabetic drug for over 50 years. It is the first-line drug of choice for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function. Metformin has pleiotropic actions, including inhibition of the mitochondrial respiratory chain complex I (El-Mir et al., 2000) and activation of the adenosine monophosphate-activated kinase (AMPK) (Zhou et al., 2001). In addition to its glycemia-lowering effects, metformin has been shown to reduce inflammation and fibrosis in a model of non-alcoholic steato-hepatitis (Kita et al., 2012). However, the effect of metformin on inflammation and fibrosis in the kidney has not been studied.

In this study, we studied the effect of treatment with metformin, initiated 1 day before surgery, on kidney structure, inflammation and fibrosis in mice with unilateral ureteral obstruction.

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2. Materials and methods

2.1. Mouse model of unilateral ureteral obstruction (UUO)

Five- to 7-month-old adult male C57Bl/6 mice, weighting 25–35 g, were anesthetized with isoflurane/oxygen and UUO was performed using 4-0 silk in the midline abdominal incision and sham-operated mice had their ureters exposed and manipulated but not ligated (Cachat et al., 2003). After 7 or 14 days of obstruction, animals were sacrificed by cervical dislocation and kidneys were decapsulated and weighed. One piece of a kidney was removed for histologic and immunohistochemical studies and the remaining was frozen in liquid nitrogen and stored at –80 °C for protein and RNA extraction. Three groups were used: sham (n = 9), sham-operated mice receiving vehicle (water) by gavage, UUO (n = 9), mice subjected to unilateral ureter obstruction model and received vehicle by gavage; and UUO + metformin (n = 10), UUO mice receiving metformin by gavage (200 mg/kg/day). All experiments were approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee.

2.2. Renal histology and Sirius Red staining

Briefly, kidneys were fixed in 10% formaldehyde and embedded in paraffin, and 4-µm sections were cut. Staining with Periodic Acid Schiff (PAS) and Masson's Trichrome were performed by the Cancer Therapy and Research Center at the University of Texas Health Science Center San Antonio. Images were obtained with an Olympus AX70 microscope using the DP image acquisition software.

2.3. Immunohistochemistry

Immunohistochemistry was performed as described by Day et al. (2013).

2.3.1. Fibronectin staining

Antigen retrieval from paraffin-embedded sections (4 µm) was performed in citrate buffer at 100 °C for 6 minutes, slides were quenched in 3% hydrogen peroxide for 6 minutes. After blocking with Background Sniper for 20 minutes, slides were incubated with the primary antibody (listed in Supplementary Fig. S1) overnight at 4 °C in a humidified chamber. After rinsing, slides were incubated with goat anti-rabbit polymer-horseradish peroxidase (Biocare, Concord, CA) for 20 minutes at room temperature. Immunoreactivity was visualized with 3-3′-diaminobenzidine (DAB, Biocare, CA).

2.3.2. F4/80, α-SMA and E-cadherin staining

Antigen retrieval from paraffin-embedded sections (4 µm) was performed in citrate buffer at 100 °C for 6 minutes. Primary antibodies (listed in Supplementary Fig. S1) were incubated overnight at 4 °C in a humidified chamber. After rinsing, slides were incubated with biotinylated secondary antibody for 20 minutes at room temperature. Streptavidin–peroxidase complex (LASB®+ System-AP, DAKO) was used for amplification and immunoreactivity was visualized with Fast (DAKO).

2.4. Morphometric analysis

Measure of tubular lumen area was performed on slides stained with E-cadherin. Images were imported in ImageJ64 and the lumen contour was traced using the free hand tool. Perimeter and surface area of the trace were determined by the software. Both perimeter and surface area show similar results, and we chose to present only the lumen surface area data, expressed in percentage of lumen area in sham-operated mice. We measured at least 15 tubules in 3–4 different mice for each group.

2.5. Reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

RNA was extracted from kidney cortex (~50 µg of tissue) using Trizol (Invitrogen), treated with nuclease I and used for reverse transcription (RT) using iScript RT SuperMix (BioRad). The resulting cDNAs were used for quantitative PCR using the RT^2 qPCR Master Mix and primers listed in Supplementary Fig. S2. The qPCR was run in a MasterCycler RealPlex4 (Eppendorf). Quantitation of the mRNAs was performed using the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

2.6. Immunoblot analysis

Slices of kidneys were homogenized in lysis buffer (Invitrogen, #FNN-0071) supplemented with protease inhibitor mix (Sigma, P-2714), 1mM PMSF, and 5 mM orthovanadate. Protein concentration was measured and 10–20 µg of whole-cell lysates was separated on SDS–PAGE, transferred to nitrocellulose membranes and probed overnight at 4 °C with the antibodies listed in Supplementary Fig. S1. IRDye800- or IRDye700-coupled secondary antibodies were used for detection using Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE).

2.7. AMPK activity

Briefly, AMPKα was immunoprecipitated from kidney homogenates using a specific antibody. Immunocomplexes were collected by centrifugation after incubation with protein A/G-agarose beads. After washes in lysis buffer and washes in PBS, the beads were resuspended in kinase buffer consisting of 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 1 mM orthovanadate and 0.05 % Triton X100. Reaction was initiated by addition of 50 µM ATP, 1 µg SAMS peptide and 10 µCi γ[^{32}P]-ATP and lasted 10 min. An aliquot of the reaction mixture was spotted on P81 paper disks which were then washed three times in 0.5% phosphoric acid and once in acetone. Radioactivity incorporated in the SAMS peptide was counted in a Beckman 6000IC scintillation counter. Antibodies used are listed in Supplementary Fig. S1.

2.8. Statistics

Data were expressed as mean ± standard error of the mean (SEM) and analyzed by analysis of variance (ANOVA) for comparison among multiple groups using the Tukey post-test analysis for comparison, using the GraphPad Prism 5.0 software. Values of $p < 0.05$ (after multiple comparison adjustment as needed) were considered significant.

3. Results

3.1. Renal morphology

Unilateral ureter obstruction was performed in 5- to 7-month-old mice. Kidney morphology was studied after 1 week of obstruction. Renal histology was studied after Periodic Acid Schiff staining of paraffin-embedded kidney sections (Fig. 1A). Obstructed kidneys displayed dilated tubules with areas of infiltrating cells (arrow), suggesting inflammation. Glomeruli were histologically normal. Metformin treatment significantly reduced the number of infiltrating cells in obstructed kidneys. E-cadherin is a transmembrane protein involved in cell–cell contact and expressed in epithelial cells (Okada, 1988). In sham-operated kidneys, E-cadherin was highly expressed in some tubules, and its expression was significantly lower in obstructed kidneys (Fig. 1B), indicating loss of tubular structure. Decrease of E-cadherin expression was attenuated