Metformin prevents renal interstitial fibrosis in mice with unilateral ureteral obstruction

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A B S T R A C T

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.

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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 fibronectin, 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.
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 RT² 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−ΔΔCT 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), 1 mM 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 MgCl₂, 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[32P]-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...
Fig. 1. Effect of metformin on renal histology and extracellular matrix expansion. (A) Renal histology was studied by Periodic Acid Schiff staining on section of paraffin-embedded kidneys. 200× magnification. (B) Extracellular matrix expansion was studied by Masson’s Trichrome staining. 100× magnification. N = 4 mice per group.

Fig. 2. Effect of metformin on inflammation in obstructed kidneys. (A) F4/80-positive cells were detected by immunohistochemistry on sections of paraffin-embedded kidneys. Positive cells stain brown. 200× magnification. N = 4 mice per group. (B) TNFα and VCAM1 expression was measured by immunoblot on homogenates of the left kidney from sham-operated, UUO and UUO mice treated with metformin. The lower panels show the results of densitometric analysis of the immunoblots. TNFα mRNA (C) and VCAM1 mRNA (D) expression was measured by RT-qPCR and normalized using GAPDH as a housekeeping gene. N = 6-9 mice per group. *p < 0.05, **p < 0.01 and ***p < 0.001 vs sham by ANOVA, ##p < 0.01 vs UUO by ANOVA.
by treatment with metformin in obstructed kidneys (Fig. 1B). Next,
we measured the tubular lumen area by morphometry. We found
that the lumen area increased about 5-fold in mice with UUO.
Metformin treatment tended to decrease the lumen surface, but the
difference did not reach statistical significance. These data show that
metformin slows down infiltration of circulating cells and tubular
damage, with a minimal effect on tubular dilatation in obstructed
kidneys.

3.2. Renal inflammation

The PAS staining detected inflammatory cells inside the paren-
chyma of obstructed kidneys. To assess whether these cells contain
macrophages, staining for F4/80 was performed. Fig. 2A shows that
few F4/80 positive cells were present in kidneys from sham-
operated mice. The number of F4/80 positive cells was significantly
increased in obstructed kidneys, mostly in the tubulo-interstitial
space. Treatment with metformin prevented the increase of F4/80
positive cells in obstructed kidneys. These data suggested that
metformin slows down macrophage infiltration in obstructed
kidneys.

TNFα plays an important role in renal inflammation in several
models of kidney injury, including UUO (Guo et al., 1999). TNFα ex-
pression was increased at the protein (Fig. 2B) and the mRNA level
(Fig. 2C) in kidneys from mice with UUO compared to sham-
operated mice. Metformin treatment prevented increased TNFα
protein expression, which remained higher than that in sham-
operated mice. TNFα mRNA levels were completely normalized by
metformin treatment (Fig. 2C). These data suggest that metformin
prevents the increase in TNFα gene transcription, but not a post-
transcriptional regulation of TNFα protein in obstructed kidneys.
Infiltration of inflammatory cells in the kidney requires coordi-
nated expression of several pro-inflammatory molecules, such as
VCAM1 which allows adhesion to the endothelium and infiltra-
tion into the renal parenchyma. VCAM1 expression was increased at
the protein (Fig. 2B) and mRNA level (Fig. 2D) in obstructed
kidneys. Metformin attenuated the increase in VCAM1 expression
in obstructed kidneys, but both mRNA and protein were still higher
in kidneys from mice UUO-metformin that in sham-operated mice.

3.3. Renal fibrosis

Unilateral ureteral obstruction causes progressive accumula-
tion of extracellular matrix in the kidney, leading to tubulo-
interstitial fibrosis. Masson’s Trichrome stain allows detection of
extracellular matrix in tissues, as blue staining. Fig. 3A shows that
obstructed kidneys have significant tubulo-interstitial and glomeru-
lar accumulation of extracellular matrix. Metformin treatment
reduced accumulation of extracellular matrix in obstructed kidneys
(Fig. 3A).

Collagens and fibronectin are two major constituents of extra-
cellular matrix in the kidney. Fibronectin deposition was assessed
by immunohistochemistry: basal staining of fibronectin in the glom-
eruli and the tubulo-interstitial compartment in kidneys from sham-
operated mice was significantly increased in obstructed kidneys
(Fig. 3B). Treatment with metformin reduced accumulation of
fibronectin deposition in obstructed kidneys. Fibronectin expres-
sion was quantified by immunoblot (Fig. 3C) and RT-qPCR (Fig. 3D).
Fibronectin protein and mRNA were upregulated in obstructed kidneys, and treatment with metformin reduce attenuated the increased fibronectin expression in obstructed kidneys.

Collagen deposition was assessed by Sirius Red staining using direct light, which allows detection of collagens I, III and IV (Fig. 4A), and polarized light, which allows detection of cross-linked collagens I and III (Fig. 4A). Collagen deposition was increased around the glomeruli and in the tubulo-interstitial compartment of obstructed kidneys. No increase staining inside the glomeruli was observed. Treatment with metformin attenuated deposition of collagens in obstructed kidneys. Expression of collagen Iα2 (Fig. 4B) and IIIα1 mRNA (Fig. 4C), measured by RT-qPCR, was increased in obstructed kidneys and normalized after treatment with metformin.

Transforming Growth Factor β (TGFβ) is an important regulator of fibrosis in several models of kidney injury (Kaneto et al., 1993; Sato et al., 2003), including the UUO. Its expression was quantified by immunoblot (Fig. 5A) and RT-qPCR (Fig. 5B). TGFβ protein and mRNA were upregulated in obstructed kidneys compared to sham-operated kidneys. Treatment with metformin prevented increased expression of TGFβ protein and mRNA.

Activation of interstitial fibroblasts plays a role in the development of tubulo-interstitial fibrosis in mice with UUO (Chevalier, 1999; Grande and López-Novoa, 2009). Fibroblast activation is accompanied by upregulation of α-smooth muscle actin (α-SMA) (Grande and López-Novoa, 2009). In kidneys from sham-operated mice, α-SMA was localized mainly in the blood vessels, and around the glomeruli some tubules (Fig. 5C). In obstructed kidneys, α-SMA was significantly increased in the tubulo-interstitial compartment, and treatment with metformin prevented this increase. The expression of α-SMA was quantified by immunoblot. Fig. 5D shows that α-SMA was increased 3.7-fold in obstructed kidneys compared to sham-operated kidneys. Metformin reduced the increase in α-SMA expression in obstructed kidneys.

Together, these data show that metformin prevents the development of renal fibrosis in obstructed kidneys, most likely through inhibition of TGFβ expression.

3.4. Adenosine monophosphate-activated kinase (AMPK)

Metformin is an indirect activator of AMPK (Viollet et al., 2012). We measured AMPKα activity by in vitro kinase assay using the SAMS peptide as a substrate (Fig. 6). Renal AMPKα activity was unchanged in obstructed kidneys, but treatment with metformin resulted in a significant increase in AMPKα activity.

4. Discussion

Our study shows for the first time that metformin treatment, initiated 1 day before surgery, attenuates development of renal inflammation and fibrosis but not of renal structural changes in mice with unilateral ureteral obstruction. The latter is a consequence of urine reflux into the kidney due to the obstruction, and is logically not affected by metformin. The former complications are due to the release of soluble factors by the injured kidney tissue. Our data suggest that this is antagonized by metformin. This is the case for TNFα, which is involved in inflammation and TGFβ, involved in fibrosis.
Metformin has been shown to have anti-inflammatory and anti-fibrotic effects in other tissues. The anti-inflammatory effects of metformin have been demonstrated in vivo in the liver and lung. Metformin reduced expression of pro-inflammatory and pro-fibrotic genes in a mouse model of non-alcoholic hepatic steatosis (Kita et al., 2012), and reduced inflammation in the airways of asthmatic mice (Park et al., 2012). The anti-inflammatory effect of metformin could be due to inhibition of the NF-κB pathway, as evidenced in cultured cancer cells (Hirsch et al., 2013), vascular cells (Isoda et al., 2006), monocytes (Arai et al., 2010) and macrophages (Hyun et al., 2013). The anti-fibrotic effect of metformin has been characterized mostly in the cardiovascular system. Metformin reduced expression of pro-fibrotic genes in hearts of mice with pressure overload (Xiao et al., 2010), in hearts of obese rats (Burlá et al., 2013), and in hearts from spontaneously hypertensive rats (Cittadini et al., 2012). The mechanism is likely through antagonism of TGFβ signaling, as demonstrated in cultured cardiac fibroblasts (Xiao et al., 2010) and canine kidney epithelial cells (Cufí et al., 2010). Our study shows for the first time that metformin exerts similar anti-inflammatory and anti-fibrotic effects in the kidney from mice with UUO.

Our data show that amelioration of inflammation and fibrosis in kidneys from metformin-treated mice was accompanied by increased activity of AMPK. Metformin has been shown to activate AMPK in several organs (Viollet et al., 2012), and although there are instances when the effects of metformin are independent of AMPK (Ben Sahra et al., 2011; Kalender et al., 2010), activation of AMPK is widely believed to mediate the effects of metformin (Viollet et al., 2012). It is therefore likely that metformin attenuates inflammation and fibrosis in obstructed kidneys through activation of AMPK. In support of this hypothesis, it has been shown that pharmacological activation of AMPK reduces fibrosis in rats with kidney ablation and infarction (Satriano et al., 2013). Our data show that AMPK activity is unchanged in obstructed kidneys, suggesting that
inflammation and fibrosis in these kidneys are due to an inhibition of AMPK activity, in contrast with diabetic nephropathy. In kidneys from rats with early diabetes, AMPK activity is decreased and pharmacological activation of AMPK reduces extracellular matrix protein expression (Lee et al., 2007).

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.06.006.

References


