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Fasudil prevents calcium oxalate crystal deposit and renal fibrogenesis in glyoxylate-induced nephrolithic mice

Haiyan Hu [1], Wei Chen [1], Jiarong Ding, Meng Jia, Jingjing Yin, Zhiyong Guo *

Department of Nephrology, Changhai Hospital, Second Military Medical University, Shanghai, 200433, China

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ABSTRACT

Nephrolithiasis is a common kidney disease and one of the major causes of chronic renal insufficiency. We develop and utilize a glyoxylate induced mouse model of kidney calcium oxalate crystal deposition for studying the pharmacological effects of fasudil, a Rho associated protein kinase (ROCK) specific inhibitor, on the kidney injury and fibrosis caused by calcium oxalate crystallization and deposition. Glyoxylate was administrated intraperitoneally to C57BL/6J mice for five consecutive days to establish a mouse model of kidney calcium oxalate crystal formation and deposition. The results showed that the protein expression levels of E-cad and Pan-ck were lower, and the protein expression levels of α-SMA and Vim were higher, in the kidney tissue of the glyoxylate induced model mice compared with the control mice. The changes in protein expression were weakened when the animals were pretreated with fasudil before glyoxylate administration. Expression of ROCK, PAI-1, and p-Smad proteins in the kidney tissue increased in response to glyoxylate treatment, and the increase was eased when the animals were pretreated with fasudil. Expression of Smad2 and Smad3 in the kidney tissue remained unchanged after glyoxylate administration. Cell apoptosis and proliferation in the kidney cortex and medulla were enhanced in response to the glyoxylate induced calcium oxalate crystal formation and deposition, and fasudil pre-treatment was able to attenuate the enhancement. The results suggest that Fasudil reduces the glyoxylate induced kidney calcium crystal formation and deposition and slows down the kidney fibrogenesis caused by calcium crystal deposition. The possible mechanism may be related the regulatory effects on Rho/ROCK signal transduction and epithelial–mesenchymal transition (EMT).

1. Introduction

One of the common causes of obstructive nephropathy is urolithiasis. With rapid improvement of the living standards, the worldwide incidence of nephrolithiasis has also risen continuously. Currently, the incidence of urolithiasis is as high as 5% to 15% of the world population and about 80% of urolithiasis is calcium oxalate related (Moe, 2006). Although the renal tubular blockade by the deposition of calcium crystals can be relieved by various medical treatments, the patient's renal function could still be severely affected due to tissue damage caused by the stone.

The pathogenesis of kidney diseases caused by calcium crystal formation and deposition still remains unclear. The decline in renal function is frequently accompanied with interstitial fibrosis which is a common pathological change of many kidney diseases. It is been known that the transition of renal epithelial cells to mesenchymal cells is a key step in the initiation and progression of renal interstitial fibrosis (Kanlaya et al., 2013; Liu et al., 2013). Epithelial–mesenchymal transition (EMT) considered that epithelial cells lose their epithelial features and acquire mesenchymal characteristics (Greenburg and Hay, 1982; Kalluri and Eric, 2003; Nishitani et al., 2005; Zeisberg et al., 2001). Occurrence of EMT in tubular epithelial cells was thought to be launched mostly by TGF-β1 (Border and Noble, 1997; Fukuda et al., 2001a, 2001b). TGF-β1 adjust cell's increment, difference and apoptosis by Smad pathway and others signal transduction such as Rho/ROCK, ERK, PI3K/Akt, JNK/p38, WNT/GSK3/β-catenin. Smad was thought to be TGF-β1's classic pathway, which has been studied more clearly. But with further researches, more and more evidence reveal that other pathway was involved in TGF-β signal transduction.

The Rho/ROCK signaling pathway is involved in the regulation of cytoskeletal protein synthesis, degradation, movement and contraction. It also plays important regulatory roles in cell division, adhesion, migration, and secretion (Riento and Ridley, 2003). Rho/ROCK signal transduction is closely related to muscle actin microfilament skeleton adjustment, some research has indicated that Rho has key function over TGF-β1 inducing renal tubular epithelial cells to transfer to myofibroblastic-like cell (Bhowmick et al., 2001; Tian et al., 2003). Fasudil is currently the only applied ROCK inhibitor in both clinic

* Corresponding author at: Department of Nephrology, Changhai Hospital, Second Military Medical University, No. 168 Changhai Road, Shanghai, 200433, China.
E-mail address: drguozhiyong@163.com (Z. Guo).
[1] These authors contributed equally to this work.

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and experiment, which can, by preventing Rho kinase, release the prevention function to MLCP and antagonism inflammatory substance vasoconstriction (Wettschureck and Offermanns, 2002), meanwhile can both promote NO creation and improve blood viscosity to dilate blood vessel (Rikitake et al., 2005), and then to improve organism's micro-circulation; besides, it can, by inhibiting inflammatory cells transference and invasion, inhibit cellular apoptosis and protein hydrolysis to alleviate organism damage (Wang et al., 2005).

Previous studies have demonstrated that Rho/ROCK-specific inhibitors are able to slowdown the development and progression of the fibrosis in cardiovascular and cerebra-vascular diseases (Loirand et al., 2006). The current work was undertaken to examine the histological and biochemical changes of kidney tubular epithelial cells that occur during the early stage of calcium oxalate crystal formation and deposition, and to assess the pharmacological effects of fasudil, a ROCK specific inhibitor, on the kidney injury and fibrosis caused by calcium oxalate crystal formation and deposition. The possible mechanism of action of fasudil was also explored and discussed.

2. Materials and methods

2.1. Experimental animals and protocols

All experimental procedures were carried out in accordance with protocols approved by the Animal Care Committee of Changhai Hospital, affiliated with the Second Military Medical University, Shanghai, China. C57BL/6J mice (7-week-old and weighing 25–28 g) were purchased from SHANGHAI SLAC LABORATORY ANIMAL CO. LTD, Shanghai, China. Fasudil purchased from ApexBio (A5734). The mice were divided into seven groups with eight animals in each group: Gly-D0, Gly-D1, Gly-D3, and Gly-D5 (with intraperitoneal injection of glyoxylate, 100 mg/kg, for 0, 1, 3, and 5 consecutive days respectively); NS (with intraperitoneal injection of 0.9% NaCl for 5 days); Gly + NS (with intraperitoneal injection of 0.9% NaCl for 5 days prior to glyoxylate administration); Gly + F (with intraperitoneal injection of fasudil 40 mg/kg for 5 days prior to glyoxylate administration) (Chart 1). 24 h after the treatment, mice were euthanized and both kidneys were surgically removed. One kidney of each mouse was fixed and stored in 4% paraformaldehyde solution. The cortex and medulla of the other kidneys were separated immediately, frozen in liquid nitrogen immediately, and stored at −80 °C until use. Glyoxylate sodium salt was purchased from Tokyo Chemical Industry, Tokyo, Japan.

2.2. Histomorphological studies

The paraformaldehyde fixed kidney tissue samples were paraffin embedded according to previous study (Boonla et al., 2011). Sections of 3-μm thickness were cut transversely and stained with Von Kossa's method for determining the calcium crystal formation and deposition. The sections were also stained with Sirius Red for collagen quantification. The Von Kossa kit used in the present work was purchased from JieMei Gene, Shanghai, China. Sirius Red was purchased from Rongbai Biotechnology, Shanghai, China.

Multiple staining procedures with immunofluorescence and immunohistochemical methods were employed to determine the protein expression levels of E-cad, Pan-ck, Vim, α-SMA, and PAI-1. PCNA and TUNEL assays were conducted to study cell proliferation and apoptosis. Antibodies against Pan-ck, Vim, α-SMA, PAI-1, PCNA, and β-actin were purchased from Santa Cruz Biotechnology, Inc. Dallas, TX, USA and those against Smad2, Smad3, p-Smad2, and p-Smad3 were purchased from Cell Signaling Technology, Danvers, MA, USA. An antibody against E-cad was purchased from Abcam, Cambridge, MA, USA. Secondary antibodies were purchased from Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA. TUNEL Apoptosis Detection Kit from Merck, Whitehouse Station, NJ, USA was used for TUNEL assay.

2.3. Western blot analysis

Western blotting experiments were conducted to determine the protein expression levels of E-Cad, Pan-ck, Vim, α-SMA, ROCK1, PAI-1, Smad2, Smad3, p-Smad2, and p-Smad3 with frozen kidney tissue samples. 80 mg of each tissue sample was lysed, separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes. After blocked with 5% skim milk at 37 °C for 1 h, the membranes were incubated with various antibodies overnight at 4 °C, and then with an appropriate horse radish peroxidase-conjugated secondary antibody for 1 h. Bound second antibody molecules were visualized with chemiluminescence detection methods. The Image-pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to determine the relative optical density using β-actin bands as standards.

2.4. Real-time PCR

mRNA was isolated from the frozen kidney samples for real-time PCR analysis. Real-time PCR was performed using a commercial kit from TaKaRa Bio Inc., Dalian, China. All of primers were designed and synthesized by Sangon Biotech Inc. Shanghai, China. And the sequences of primers were listed in Table 1.

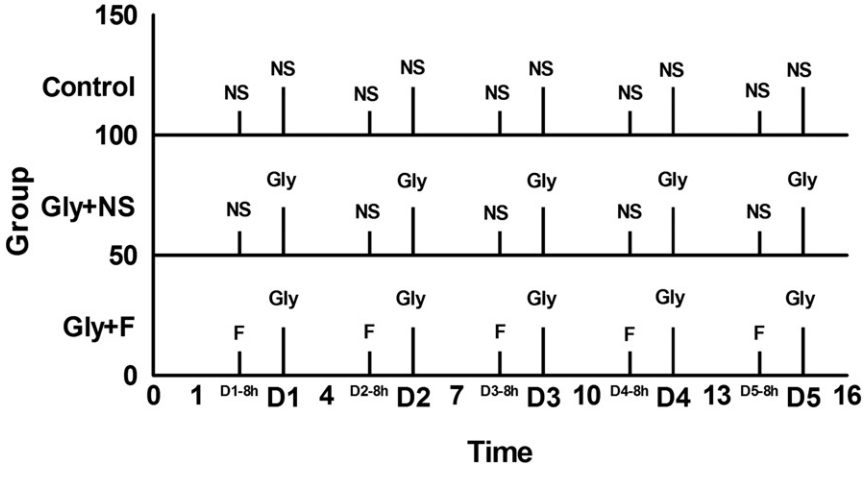

Chart 1 The flow chart of animal experiment.