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Effects of asiaticoside on levels of podocyte cytoskeletal proteins and renal slit diaphragm proteins in adriamycin-induced rat nephropathy ☆

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ABSTRACT

Aims: Centella asiatica has been used to treat kidney diseases in Chinese traditional medicine. Asiaticoside (an extraction of *C. asiatica*) exerts a variety of pharmacological effects including immunomodulatory and anti-inflammatory functions. However, the mechanism of asiaticoside in the treatment of renal diseases remains largely unknown. This study investigated the molecular mechanism of asiaticoside in treating adriamycin-induced nephropathy of rats.

Main methods: Sixty-two SD male rats were randomly divided into normal control group (n = 12) and nephropathy group (n = 50). Except for the normal control group, rats were injected with adriamycin (6 mg/kg) via the tail vein to induce nephropathy. Adriamycin induced nephropathic rats were divided into untreated group, prednisone group (25 mg/kg), and asiaticoside groups with various dosages (8, 16 and 32 mg/kg). Samples of urine and serum, tissue of kidney were collected for analysis after treatments for four weeks. Morphological changes were evaluated under light microscope and electron microscope. Synaptopodin, desmin, nephrin and podocin mRNA and protein were determined by RT-PCR and Western blotting.

Key findings: Compared to the untreated nephropathy group, asiaticoside treatment mitigated histological damages, decreased 24-hour urine protein excretion and total cholesterol, increased serum albumin. Asiaticoside treatment increased the mRNA and protein levels of synaptopodin, nephrin and podocin in a dose-dependent manner. Furthermore, asiaticoside treatment decreased the mRNA and protein levels of desmin.

Significance: Asiaticoside can mitigate adriamycin-induced nephropathy in rats, which is associated with the increase in synaptopodin, nephrin and podocin gene expression, and the decrease in desmin gene expression.

Introduction

Proteinuria is one of the most common features of renal disease and is a consequence of problems with the glomerular filtration barrier. The glomerular filtration barrier is composed of capillary endothelial cells, glomerular basement membrane (GBM) and the slit diaphragm located between the foot processes of podocytes. Since the fenestrae of endothelial cells have large diameters, filtration of the protein by this portion of the barrier is limited. The GBM has a surface rich with anionic charges and functions as a charge barrier, and is composed of fibers arranged in a fine sieve-like network. Podocytes are located outside of the GBM and are divided structurally and functionally into three portions: cell body, major processes, and foot processes. Microtubules maintain and alter the morphology of podocyte foot process by arranging an actin-based cytoskeletal system. Adjacent podocyte foot processes interdigitate, with interstices bridged by a slit diaphragm (SD). The SD filtration membrane barrier is the most important portion of the large filtration barrier (Ishizuka et al., 2007). Podocytes are an end-stage differentiated cell, lacking regenerative capacity following abscission (Kerjaschki, 2001). They comprise the last barrier against the protein loss from the body. Once podocytes are injured, proteinuria develops. The special structure of podocytes mainly depends on the expression of cytoskeletal system components and a number of specific proteins. These specific protein molecules and proteins of the slit diaphragm are important portions of the podocyte barrier, regulating normal renal function.

The adriamycin-induced nephropathy model was reported in the early 1980s and has been widely used in the field of kidney disease. Adriamycin is a quinone-containing anthracycline antibiotic and can be reduced to a semiquinone radical by metabolism in the kidney. The latter reacts with oxygen to produce reactive oxygen, inducing lipid peroxidation in the glomerular epithelial cells and destruction of the structure and function of the filtration membrane and ultimately proteinuria (Oka et al., 2008). An acute adriamycin-induced nephropathy model is induced by a single tail vein injection of 5–7.5 mg/kg adriamycin. This model similar to human minimal change nephrotic

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syndrome has been well characterized as an experimental model for nephrotic syndrome.

Centella asiatica is a member of the umbelliferae group of plants, and may reduce swelling, stimulate circulation, and promote diuresis (Qi and Wang, 2010). *C. asiatica* has been used to reduce urinary protein excretion in the treatment of kidney diseases for thousands of years in China. The chemical components of *C. asiatica* include triterpenoid saponins, triterpene acids, polyacetylene alkenes and volatile oils (Chen and Sun, 2006). Asiaticoside is widely used clinically for its pharmacological properties, such as immunomodulatory, anti-inflammatory, and antiviral properties, as well as promotion of wound healing (Yang, 2008). However, the exact mechanism of action of asiaticoside in the treatment of renal diseases remains largely unknown. The present study was designed to examine therapeutic roles of asiaticoside in adriamycin-induced nephropathy in rats. Specifically, effects of asiaticoside on expression of synaptopodin, desmin, nephrin and podocin were examined.

Materials and methods

Animals

Sixty-two male healthy SPF-grade SD rats (150 ± 20 g) were provided by the Experimental Animal Center of the School of Medicine, Xi'an Jiaotong University. Rats were housed under conditions of constant temperature (21 ± 2 °C) and humidity (60%) and exposed to a 12-h dark-light cycle. All rats were fed on a standard pellet diet and water was freely available.

Experimental design

Rats were randomly divided into a normal control group (Group A, n = 12) and a nephropathy model group (n = 50). The nephropathy model was prepared as described previously (Ren et al., 2010). Rats were injected with adriamycin (6 mg/kg, single dose) via the tail vein to induce nephropathy. The control group rats were injected with saline. Nephropathy was confirmed after three weeks by massive proteinuria, hypoalbuminemia, hyperlipidemia as well as by foot process effacement illustrated by electron microscopy (at the end of seventh week). Forty nephropathy rats were randomly divided into untreated nephropathy model group (Group B), asiaticoside groups (Groups C, D, and E, n = 8, respectively) and a positive control group (Group F, n = 8). Prednisone was used as a positive control (Gipson et al., 2009). From the 4th week on, Groups A and B were administered saline; Groups C, D, and E were administered 8 mg/kg, 16 mg/kg, and 32 mg/kg asiaticoside (Zhang et al., 2007), respectively; Group F was administered 25 mg/kg prednisone. All drugs and saline were administered by gavage once a day for 4 weeks.

At the end of the 3 and 7 weeks after the first adriamycin injection, 24 h urine samples were collected by metabolic cages; blood was collected from the abdominal aorta, and serum was prepared via centrifugation and stored at −20 °C. At the end of the 7 weeks, all rats were euthanized. Parts of fresh kidney tissues were removed and fixed in 10% formaldehyde for routine histological examination; whereas another part of the kidney tissues was fixed in 2% glutaraldehyde for transmission electron microscopy examination. The remaining kidney tissues were kept in liquid nitrogen for protein and mRNA measurements. All animal procedures were conducted in accordance with the China Animal Welfare Legislation and were approved by the Ethics Committee on the Care and Use of Laboratory Animals in Xi'an Jiaotong University (Shaanxi, China).

Biochemical analyses

Twenty four-hour urine protein excretion was measured by pyrogallol red-molybdate method (DiaSys Diagnostic Systems Co., Ltd, Shanghai). The levels of albumin (ALB), serum triglyceride (TG), total cholesterol (TC), urea nitrogen (BUN), and creatinine (Cr) were detected by Automatic Analyzer (7600-020, Hitachi, Tokyo, Japan).

Histological examination

The fixed tissue specimens were dehydrated in graded alcohol solutions, cleared in toluene, and embedded in paraffin. Sections (3 μm) were stained with periodic acid-Schiff stain (PAS). For the electron microscopic analysis, $1 \times 1 \times 1\ mm^3$ fragments of the renal tissues were fixed overnight in cold glutaraldehyde (2%) in 0.1 M sodium cacodylate buffer for 2 h in 4 °C. After dehydration in graded ethanol, the pellets were embedded in Spurr. Ultra-thin sections were observed with the electron microscope (H600A, Hitachi, Tokyo, Japan).

Real-time-polymerase chain reaction (PCR)

Total RNA was purified from fresh rat kidney cortex tissue (50–100 mg) on ice with RNAfast1000 (Pioneer, China). The purity of total RNA was checked with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.7 and 2.0 in all preparations. Reverse transcription of total RNA (0.1–5 μg) to cDNA was carried out using RevertAid First Strand cDNA Synthesis Kits (MBI, Lithuania) in 20 μL volume reaction at 44 °C for 1 h by using Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany). Specific primers were designed using Beacon Designer 4.0 software and synthesized with Sunbiotech (Beijing Sunbiotech CO., Ltd, China). Real-time PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (2×) (MBI, Lithuania) in the IQ5.0 system (BioRad, USA). The system automatically monitors the binding of a fluorescent dye SYBR® Green to double-stranded DNA during each cycle of PCR amplification. PCR was performed under the following conditions: Heating 50 °C for 2 min, followed by 95 °C for 10 min and 40 PCR cycles with 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. The relative amount of mRNA was calculated using the comparative C_t ($\Delta\Delta C_t$) equation. The β-actin gene was used as a reference for normalizing data. Derived normalized values are the mean of three runs. The sequences of the rat and human primers (Beijing Sanbo Yuanzhi Biotechnology, Ltd) are listed in Table 1.

Western blotting

Kidney tissue homogenates or cells were lysed by RIPA (radioimmunoprecipitation assay) lysis buffer (Pioneer, China), which enables the extraction of cytoplasmic, membrane and nuclear proteins. Samples containing 100 μg of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane. Nonspecific binding was blocked with 5% non-fat dry milk in 0.1% Tween-20/TBS (TBST) for 1 h at room temperature. Following washing with TBST, the blots were incubated overnight at 4 °C with the primary antibody: synaptopodin, 1:500 (Proteintech, USA), desmin, 1:1000 (Proteintech, USA), podocin, 1:200 (Proteintech, USA), nephrin, 1:200 (Abcam, USA), and β-actin, 1:2000 (Santa Cruz Biotechnology, USA). The membranes were incubated for 1 h at room temperature with the corresponding HRP-conjugated secondary antibodies (synaptopodin, desmin, podocin, and nephrin were goat anti-rabbit IgG(H + L); β-actin was goat anti-mouse IgG(H + L), Pierce, USA). Signals were visualized using a GE

Table 1
Real-time PCR primer sequences.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
Synaptopodin	CCACAGAGGCACATAATG	GGATACAGAGTAGAATAAGAGG
Desmin	TCCTACACCTGCGAGATTG	GCGATGTTGTCCTGATAGC
Nephrin	TTCTGCTGCTCTCCAATG	ACTTCTGCTGTGCTAACC
Podocin	GGTGATTGCTGCCGAAGG	CTGTGGACAAGGACTGAAGAG