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Original article

Paeoniflorin ameliorates Adriamycin-induced nephrotic syndrome through the PPARγ/ANGPTL4 pathway *in vivo* and *vitro*

Ruirui Lu[1], Jie Zhou[1], Bihao Liu, Ning Liang, Yu He, Lixia Bai, Peichun Zhang, Yanchun Zhong, Yuan Zhou*, Jiuyao Zhou*

Department of Pharmacology, College of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou 510006, PR China

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ABSTRACT

Paeoniflorin (PF), an effective composition that is extracted from *Radix Paeoniae* Alba, plays a role in protecting against various kidney diseases. However, the mechanism of PF on nephrotic syndrome (NS) remains unclear. The aim of this study was to investigate the protective role of PF on Adriamycin (ADR)-induced NS *in vivo* and *vitro* as well as its potential mechanism. In animal study, PF significantly decreased the levels of 24-h urine protein, blood urea nitrogen, serum creatinine, total cholesterol and triglycerides in NS rats, but increased the total protein and albumin levels. Hematoxylin-eosin (HE) staining revealed that the kidney lesion was resolved upon PF treatment. After treatment with PF, the morphology and number of podocytes in renal tissue were restored to normal. PF increased expression of synaptopodin and decreased expression of desmin, demonstrating a protective effect in podocyte injury. Further studies revealed that PF upregulated Peroxisome proliferator-activated receptor gamma (PPARγ) and restrained Angiopointin-like 4 (ANGPTL4) in kidney tissue. *In vitro* study, PF reduced Caspase3 and Bax and increased Bcl-2, indicating that the apoptosis rate of podocytes induced by ADR was reduced by PF. Furthermore, PF ameliorated podocyte injury by upregulating synaptopodin and reducing desmin. In accordance with animal study, PF downregulated ANGPTL4 by activating PPARγ. However, the therapeutic effects of PF were reversed by GW9662 (PPARγ inhibitor), likely by suppressing ANGPTL4 degradation. In general, these results demonstrate that PF has a good therapeutic effect on NS by activating PPARγ and subsequently inhibiting ANGPTL4.

1. Introduction

Nephrotic syndrome (NS) is one of the primary glomerulonephritis conditions that is characterized by chronic inflammation, oxidative stress, proteinuria and generalized edema [1]. Unfortunately, NS is a life-threatening condition that affects millions of people worldwide, particularly children [2,3]. If no timely prevention and treatment methods were available, NS will eventually develop into renal failure. Thus, it is imperative to identify an effective drug treatment.

Significant proteinuria is the central link in the development of NS, which is also an important factor that affects other symptoms [4]. The formation of proteinuria is associated with glomerular filtration barrier damage [5]. As the last barrier of glomerular filtration, podocytes and their silt diaphragm play an important role in the formation of proteinuria during nephrotic syndrome. Therefore, podocytes can serve as a key target in the treatment of nephrotic syndrome [6,7].

Angiopoietin-like Protein4 (ANGPTL4), a new type of secreted glycoprotein, plays an important role in the regulation of metabolism and inflammation. Recent research demonstrates that ANGPTL4 also plays a major role in kidney disease. In the model of Hayman's disease, ANGPTL4 was highly expressed in glomerular podocytes [8]. The upregulation of ANGPTL4 secreted by podocytes can lead to the fusion of podocyte foot processes and the damage of glomerular basement membrane charge barrier, resulting in significant proteinuria [9,10]. ANGPTL4 is the target gene of peroxisome proliferator-activated receptor γ (PPARγ), which regulates ANGPTL4 expression [11,12]. PPARγ also plays an important role in kidney disease. In cultured podocytes, PPARγ agonists reduced podocyte injury and apoptosis induced by PAN [13]. Moreover, PPARγ agonists alleviated proteinuria in patients and animals with nephrotic syndrome and improved foot

Abbreviations: PF, paeoniflorin; NS, nephrotic syndrome; ADR, adriamycin; PPARγ, peroxisome proliferator-activated receptor gamma; ANGPTL4, angiopointin-like 4; HE, hematoxylin-eosin; Scr, Serum creatinine; TG, triglycerides; TP, serum total protein; ALB, serum albumin; TC, total cholesterol; BUN, blood urea nitrogen

* Corresponding authors at: Department of Pharmacology, College of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou, 232 WaiHuan East Road, Guangzhou University Town, Guangzhou 510006, China.

E-mail addresses: zygz@gzucm.edu.cn (Y. Zhou), zhoujiuyao@tom.com (J. Zhou).

[1] Both authors contributed equally to this work.

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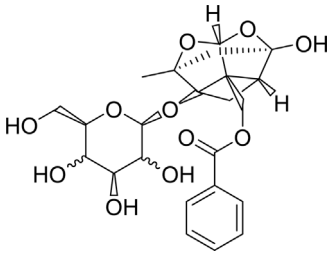

Fig. 1. Chemical structure of paeoniflorin.

process fusion in rats treated with PAN [14].

Paeoniflorin (PF) is extracted from the root of *Paeonia lactiflora*, which possesses anti-inflammation, analgesia and immune-regulation effects [15–17]. Research demonstrates that the PF has a role in the treatment of kidney disease by ameliorating renal fibrosis, acute renal injury, and diabetic nephritis [18,19]. Furthermore, study demonstrated that PF enhanced the PPARγ mRNA and protein levels that suppressed by TNF-α in adipocyte dysfunction. PPARγ agonist could ameliorate podocyte injury and reduce proteinuria in acute nephrotic syndrome [20].

However, the specific pharmacological action and mechanism of PF on NS require further study. To study the effects of PF on the progression of NS, rats were intravenously injected with a single dose of Adriamycin (ADR) as an *in vivo* model, and ADR-induced injury in podocytes was used as an *in vitro* model. The aim of present study was to determine our hypothesis that PF prevents podocytes injury in NS through PPARγ/ANGPTL4 pathway, resulting in ameliorates proteinuria in ADR-induced NS.

2. Materials and methods

2.1. Chemicals and reagents

Paeoniflorin (Fig. 1) was purchased from Li Hua Pharmaceuticals Inc. (Lot: 20140712). Tacrolimus was purchased from Sigma-Aldrich (US; Cat: 109581-93-3). Adriamycin hydrochloride was provided by Shen Zhen Wan Le Pharmaceutical Co. Ltd, Shenzhen (Lot: 0901E). The total protein extraction kit was obtained from Sigma-Aldrich, US. Coomassie (Bradford) Protein Assay Kits were purchased from Beijing Kangwei Bioengineering Institute (Lot: 50111). Pierce® BCA Protein Assay Kit (Cat: A045), Urea nitrogen reagent assay kit (Cat: C013-2), Albumin assay kit (Cat: A028-2), triglyceride assay kit (Cat: A110-2), creatinine kit, total cholesterol (Cat: A111-2), and Mayor's hematoxylin (Lot: #20140801) were all provided by Nanjing Jiancheng Bioengineering Institute. Trizol (Lot: A9902-1) and the reverse transcription kit (Lot: AK2003) were from TaKaRa, US. PV-900 Immunohistochemical assay kit (Lot: #k14771c) and DBA reagent kit (Lot: #k14771c) were the products of Beijing ZhongshanJinqiao Bioengineering Institute. Loading buffer (5×) (Lot: 2515C) was obtained from Beijing Kangwei Shiji. PCR amplification kits were purchased from bao Bioengineering Institute Dalian. Rabbit polyclonal anti-PPARγ, anti-ANGPTL4, anti-Caspase3, anti-Bcl-2, and anti-Bax were purchased from Cell Signaling Technology. Rabbit anti-Desmin and anti-synaptopodon antibodies were obtained from Santa Cruz Biotechnology, CA.

2.2. Animal model and experiment groups

Sixty adult male Sprague–Dawley rats (special pathogen-free level, Certificate No. SCXK2013-0034) weighing between 180 and 220 g were provided by The Experimental Animal Centre of Guangzhou University of Chinese Medicine. All animal experimental procedures were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine according to the guidelines of the European Community and the National Institute of Health of the USA. All rats were provided standard rat chow and tap water ad libitum and housed at 23 ± 2 °C. After 1 week of acclimatization, all the other rats were intravenously injected with a single dose of Adriamycin (6.5 mg/kg, dissolved in normal saline) with the exception of 10 rats from the normal group. The normal group was injected with normal saline. We detected 24-h proteinuria to ensure that the model was successful. Subsequently, the successful model rats were randomly divided into four groups: model group, tacrolimus (positive drug) group (1 mg/kg), PF high dose (PFH) group (200 mg/kg), and PF low dose (PFL) group (100 mg/kg). The normal group and model group were orally administered normal saline at a dose of 1 mL/100 g. All of the rats were administrated normal saline or PF or tacrolimus for 42 days. The complete experimental time was 7 weeks.

2.3. Measurement of urine protein

Briefly, 24-h urine was collected from metabolic cages once a week to measure uric protein levels. During the urine collection period, all rats were forbidden from food but provided free access to water. Coomassie (Bradford) Protein Assay Kits were used to determine urine protein levels.

2.4. Blood sampling and renal tissue removal

24-h after the last administration, all the experimental rats were euthanatized, and blood samples and renal tissue were obtained. Five milliliters of blood from the abdominal aorta were collected and then centrifuged at 3500 rpm at 4 °C for 15 min to obtain the serum for biochemical analysis. Both kidneys from each rat were divided into two parts. One part was fixed in 10% neutral formalin phosphate buffer for HE staining, and the other was quickly frozen in liquid nitrogen and stored at −80 °C for real-time quantitative PCR and Western blot analysis and as a backup.

2.5. Detection of serum biochemical indexes

Serum creatinine (Scr), triglycerides (TG), serum total protein (TP), serum albumin (ALB), total cholesterol (TC), and blood urea nitrogen (BUN) levels were tested according to instructions of assay kits.

2.6. HE staining of renal tissue

The HE staining protocol is based on our previous study. In short, the renal tissue samples fixed in 10% (v/v) neutral formalin phosphate buffer were dehydrated in a graded series of alcohol and embedded in paraffin. The 5-μm thick sections were stained with hematoxylin for 3 min, washed and then stained with 0.5% hematoxylin–eosin (HE) for an additional 3 min. The morphological changes in the kidney were blindly evaluated under a light microscope (TE2000, Nikon, Japan) by an experienced pathologist.

2.7. Observation of renal tissue by transmission electron microscope

The fresh kidney was cut into a size of approximately 1 mm^3 and immediately fixed in 5% glutaraldehyde for 2 h. Then, 1% osmic acid was added for 1–2 h. The samples were washed thrice with PBS, each time for 10 min. Dehydration was attained with different concentrations of acetone. Then, the sample was immersed into a mixture of different proportions of the dehydrating agent and the embedding agent (2:1, 1:1, and 1:2) for 1 h and finally immersed in embedding medium overnight. The infiltrated tissue was covered with epoxy resin Epon812 and placed in an oven for drying. Next, the dried tissue was cut into 50- to 70-nm slices, which were stained with lead citrate and uranyl acetate and then separated by sodium hydroxide. The sections were rinsed with double distilled water and dried, and then were examined under an electron microscope (JEM100CX-a, Japan) at 60kv, ×7500