Toxicology Reports 2 (2015) 121–129

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

# Reduced kidney levels of lysophosphatidic acids in rats after chronic administration of aristolochic acid: Its possible protective role in renal fibrosis

Toshihiko Tsutsumi[a,*], Syougo Yamakawa[b], Akira Ishihara[c], Aimi Yamamoto[b], Tamotsu Tanaka[b], Akira Tokumura[b,d]

[a] Department of Pharmaceutical Sciences, Kyushu University of Health and Welfare, Japan
[b] Department of Pharmaceutical Health Chemistry, Institute of Health Biosciences, University of Tokushima Graduate School, Japan
[c] Department of Anatomic Pathology, Prefectural Nobeoka Hospital, Japan
[d] Department of Life Sciences, Faculty of Pharmacy, Yasuda Women's University, Japan

ARTICLE INFO

*Article history:*
Received 15 December 2014
Received in revised form 30 January 2015
Accepted 23 February 2015
Available online 28 February 2015

*Keywords:*
Lysophospholipid
Aristolochic acid
Lysophosphatidic acid
Chronic kidney disease
Fibrosis
Nephrotoxicity

ABSTRACT

Aristolochic acid (AA) is considered to be a causative agent for progressive interstitial renal fibrosis, leading to AA nephropathy. Lysophosphatidic acid (LPA) is a mediator in the onset of renal fibrosis. In this study, we analyzed the molecular species of LPA and its precursor lysophospholipids in kidney tissue from rats exposed to AA. Daily intraperitoneal injections of AA for 35 days to rats gave rise to fibrosis in kidney, decreased the kidney levels of LPA, lysophosphatidylserine and lysophosphatidylinositol. In rat renal cell lines (NRK52E and NRK49F), AA-induced cytotoxicity was potentiated by Ki16425, $LPA_{1,3}$ receptor antagonist. The level of mRNA encording α-smooth muscle actin was significantly increased by AA-treatment only in NRK52E cells, while the mRNA level of collagen III was decreased in both NRK52E and NRK49F cells. These results suggest that endogenous LPA in rat kidney prevents AA-induced renal fibrosis.


## 1. Introduction

The physiological and pathophysiological roles of lysophospholipids (LPLs), especially lysophosphatidic acid (LPA), have been studied extensively in mammals. LPA is now recognized as not only an intermediate in synthesis of glycerophospholipids but also an important lipid mediator [1–3]. Recent rapid progress in liquid chromatography–tandem mass spectrometry (LC–MS/MS) with high selectivity and sensitivity has contributed greatly to the progress of biochemical, physiological and pathological studies of LPLs [4]. In a unilateral ureteral obstruction model in rats, we found higher production of LPA from lysophosphatidylcholine (LPC) in the swollen renal pelvis in the ligated kidney than in bladder urine [5]. We reported prevention by LPA of $Cd^{2+}$-induced death of cultured rat renal proximal epithelial cells (NRK52E) and interstitial fibroblast cells (NRK49F), suggesting that endogenously generated LPA protects rats from the renal toxicity of $Cd^{2+}$ [6]. However, there is no information on renal

*Abbreviations:* 18S, ribosomal protein S18; AA, aristolochic acid; α-SMA, α-smooth muscle actin; LC–MS/MS, liquid chromatography–tandem mass spectrometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; lysoPLD, lysophospholipase D; LPL, lysophospholipid; $PLA_1$, phospholipase $A_1$; $PLA_2$, phospholipase $A_2$; HE, hematoxylin/eosin; AZ, azan Mallory.

* Corresponding author at: Department of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshinomachi, Nobeoka 882-8508, Japan. Tel.: +81 982 23 5516; fax: +81 982 23 5517.
*E-mail address:* t-tsutsumi@phoenix.ac.jp (T. Tsutsumi).

http://dx.doi.org/10.1016/j.toxrep.2015.02.012

tissue level of LPA in rats that were subjected to renal injury.

Aristolochic acid (AA) is an alkaloid extracted from mainly seeds of *Aristolochia clematitis* and roots of *Aristolochia fangchi* and *Aristolochia manshuriensis*. *Aristolochia* species are used for treatment for arthritis, gout and rheumatism [7]. AA was shown to act as an analgesic, diuretic and anti-inflammatory agent [8]. However, AA is now considered a causative agent for progressive interstitial renal fibrosis and urothelial carcinoma development in the upper urinary tract. These pathological lesions developed in seemingly unrelated events. The first event occurred in the 1950s in rural villages in the drainage basin of the Danube river [9] and the second occurred in Belgium in young women who had taken slimming pills including Chinese herbs [10]. Since these events, the uses of AA-containing medicinal substances and supplements have been banned in many countries. On the other hand, AA is known to have an inhibitory effect on phospholipase $A_2$ ($PLA_2$) and to prevent inflammatory diseases, such as *Clostridium difficile*-induced diarrhea-related events [11]. Therefore, we postulated that chronic AA-induced nephropathy leads to altered levels of LPA and its related LPLs in animal body tissues. In this study, we examined whether AA-nephropathy is accompanied by altered levels of LPLs in whole kidney tissue of rats.

## 2. Materials and methods

### 2.1. Materials

AA, a mixture of AAI and AAII, was purchased from Across Organics (Geel, Belgium). Ki16425, an $LPA_{1,3}$ receptor antagonist, was obtained from Cayman Chemical (Ann Arbor, MI, USA). Sepasol RNA I super and polyethylene glycol #400 were obtained from Nakarai tesque (Kyoto, Japan). 1-Heptadecanoyl (17:0) LPC, 1-heptadecenoyl (17:1) lysophosphatidylinositol (LPI), 17:0 lysophosphatidylglycerol (LPG), 17:0 lysophosphatidylethanolamine (LPE), and 17:0 lysophosphatidylserine (LPS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 17:0 LPA was prepared from 17:0 LPC using phospholipase D from *Streptomyces chromofuscus* as described previously [12]. Egg yolk phosphatidylcholine (PC), 1-palmitoyl LPC, and 1-palmitoyl LPA as standards for TLC were obtained from Funakoshi Co. (Tokyo, Japan). Alzet™ osmotic pumps were purchased from Durect (Cupertino, CA, USA). Chow (MF) purchased from Oriental Yeast (Tokyo, Japan) had the following components: powders of wheat, defatted soybean, alfalfa, defatted rice, defatted bovine milk, soybean oil, corn, white fish meal, and beer yeast.

### 2.2. Animal experiments

Male Wistar/ST rats (4–5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The breeding room was kept at 20–25 °C with a light–dark cycle of 12 h each. Rats were allowed free access to a chow and water throughout the experimental period. All rats were handled in accordance with the principles and guidelines of the Experimental Animal Committee of Kyushu University of Health and Welfare. The rats were divided into two groups of 6 animals each in three separate experiments and acclimatized for 1 week. One week later, AA was dissolved in 100% polyethylene glycol #400 at a final concentration of 10 mg/ml and was subcutaneously (with and without Alzet™) or intraperioneally injected at 10 mg/kg body weight daily for 35 days. The sham rats were given the vehicle solution (1 ml/kg).

### 2.3. Histological analysis

After AA treatment for 35 days, kidneys were quickly removed. The kidney slices were fixed with 10% formaldehyde and then embedded in paraffin. Degrees of renal tissue injury were evaluated by microscopic observation after staining with hematoxylin/eosin (HE) and azan Mallory (AZ).

### 2.4. Lipid extraction from kidney tissue

The frozen kidney ($0.24 \pm 0.11$ g for 6 rats) was placed on ice and then de-frosted tissues were homogenized in a glass homogenizer containing 4 ml of a mixture of chloroform–methanol–water at a final proportion of 1:2:0.8 (v/v), followed by centrifugation at $1100 \times g$ for 10 min at 4 °C after standing on ice for 2 h. The precipitates were mixed with 0.5 ml chloroform, 1 ml methanol and 1 ml distilled water, and the mixture was centrifuged at $1100 \times g$ for 10 min at 4 °C. The supernatants were combined, and the pH was adjusted to 9–10 with 20% $NH_4OH$. After mixing the supernatant with 1.5 ml chloroform and 1.5 ml distilled water, the mixture was centrifuged at $1100 \times g$ for 10 min at 4 °C. The resultant upper phase was withdrawn and mixed with 3 ml of a mixture of chloroform–methanol (17:3), followed by centrifugation. The lower phases were combined for LC–MS/MS of LPC. The pH of the remaining upper phase was adjusted to 2–3 with 5 M HCl, and polar acidic LPLs were extracted twice with 3 ml of chloroform–methanol mixture (17:3). The lower phases were combined for LC–MS/MS of LPA, LPG, LPI and LPS.

Before the lipid extraction, 10 nmol 17:0 LPC, 0.5 nmol 17:0 LPS, 0.5 nmol 17:0 LPG, 0.2 nmol 17:1 LPI and 0.5 nmol 17:0 LPA were added as internal standards for corrections of efficiencies of lipid extraction and electrospray ionization. The first lipid extract was dried under a stream of nitrogen gas, and were dissolved in 0.5 ml of a mixture of methanol/water (95:5, v/v) containing 5 mM formic acid for LC–MS/MS. The second lipid extract containing acidic polar lipids such as LPA, LPS, LPG and LPI were dissolved in 0.1 ml of a mixture of methanol/water (95:5, v/v) containing 5 mM formic acid for LC–MS/MS.

### 2.5. LC–MS/MS of LPLs

LC–MS/MS was performed on a quadrupole-linear ion-trap hybrid MS, 4000 QTRAP™ (Applied Biosystems/MDS Sciex; Concord, ON, Canada), with an Agilent 1100 LC system combined with an autosampler (HTS PAL, CTC Analytics, Zwingen, Switzerland), as previously described [13]. Separation of LPCs in the neutral lipid fractions by