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Reduced rat plasma lysophosphatidylglycerol or lysophosphatidic acid level as a biomarker of aristolochic acid-induced renal and adipose dysfunctions

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ABSTRACT

Aims: Food products and diet pills containing aristolochic acid (AA) are responsible for a rapid progression of nephropathy associated with reduced body weight in human beings. In this study, we investigated the relationship of dietary NaCl and lysophospholipid (LPL) plasma levels to body weight gain in AA-treated rats.
Main methods: Male rats receiving a salt-deficient chow, normal salt chow or high salt chow were injected intraperitoneally daily with AA for 15 days. Body weight, visceral fat mass, food intake, levels of LPL in plasma and its synthesized enzyme were investigated.
Key findings: Body weight gain, visceral fat mass and daily food intake were smaller in AA-treated rats than those of control rats, regardless of dietary salt concentration. AA treatment decreased plasma levels of major lysophosphatidic acid (LPA) molecular species in rats fed the normal or high-salt chow but not the salt-deficient chow, whereas both the plasma lysophospholipase D activity and kidney mRNA level of autotaxin of AA-treated rats fed chow with defined salt concentrations were lower than those of control rats. Plasma levels of major molecular species of lysophosphatidylglycerol (LPG) in AA-treated rat groups fed chow with defined salt concentrations were lower than those of control rats.
Significance: Plasma levels of LPG and LPA seem to be relevant to the reduced body weight gain and fat mass due to AA treatment.

1. Introduction

Aristolochic acid (AA) is a naturally occurring substance in some *Aristolochia* species, and now generally accepted as a causative agent of nephropathy, which is characterized by serious anemia, progressive tubulointerstitial fibrosis, glycosuria, leukocyturia, normal to mildly increased blood pressure and elevated serum creatinine in humans [1]. AA-induced nephropathy rapidly develops to end-stage kidney failure with a high prevalence of urothelial carcinoma [2]. Natural health products containing *Aristolochia* species have been utilized to reduce obesity [3]. In the United Kingdom, intake of a Chinese herbal tea containing AA was reported to induce body weight loss [4]. AA is also used as a snake venom antidote in folk medicine due to its inhibitory effect on *in vitro* phospholipid hydrolysis by phospholipase A_2 [5].

Lysophospholipids (LPLs) such as lysophosphatidic acid (LPA) are lipid mediators, and dysregulation of their metabolisms causes various pathophysiological states, including nephropathy [6], fibrosis [7], and cancer [8]. LPA in animal plasma is generated mainly by autotaxin (ATX), an ectoenzyme with lysophospholipase D (lysoPLD) activity [9]. We previously reported lower renal levels of LPA, lysophosphatidylinositol (LPI), and lysophosphatidylserine (LPS) in rats with AA-induced nephropathy and inhibition by LPA of the AA-induced cytotoxic effect on cultured renal epithelial cells, suggesting that endogenous LPA plays a potential role in rat kidney tissue by protecting it from AA-induced renal fibrosis [10].

Dietary sodium is suggested to affect body weight, and high sodium intake increased weight gain in a human clinical study [11]. However, prolonged intake of a low-salt chow from weaning to adulthood increased the body weight of rats despite their lower food intake compared to rats fed a normal or high-salt chow [12]. In this study, we examined whether AA treatment and differences of dietary salt consumption affect plasma levels of LPLs including LPA as a possible

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circulating mediator of AA-induced nephropathy or reduced body weight gain of rats.

2. Materials and methods

2.1. Materials

AA was purchased as a mixture of AAI and AII from Acros Organics (Geel, Belgium). Sepasol RNA I Super G and polyethylene glycol #400 were obtained from Nacalai Tesque (Kyoto, Japan). Heptadecanoyl (17:0) lysophosphatidylglycerol (LPG) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 17:0 LPA was prepared from 17:0 lysophosphatidylcholine (LPC) using phospholipase D from *Streptomyces chromofuscus* as described previously [13]. The fatty acyl residue in LPLs was tentatively represented as the number of carbon chain: the number of double bond. Normal salt chow (NS) containing 0.26% sodium chloride (AIN-93G) and cornstarch, milk casein, sucrose, soya bean oil, powdered cellulose, minerals, vitamins, L-cystine, choline bitartrate, and tertiary butylhydroquinone, was purchased from Oriental Yeast (Tokyo, Japan). The AIN 93G chow with no added NaCl (LS) was used for dietary salt restriction in this study, as previously used to examine renal disturbance in rats [14]. A chow containing 4.00% NaCl (HS) was used to evaluate high salt-induced onset and aggravation of nephropathy as monitored by excretion of urinary protein and observation of glomerular injury [15,16]. LS and HS were custom-made from modified AIN-93G by Oriental Yeast.

2.2. Animal treatment and body fluid sampling

Four-week-old male Wistar/ST rats 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 the LS, NS, or HS 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 six groups of six animals each at random and acclimatized for one week. AA was dissolved in 100% polyethylene glycol #400 at a final concentration of 10 mg/ml and intraperitoneally injected at 10 mg/kg body weight daily for 15 days. The control rats were injected with the vehicle alone (1 ml/kg). Rats were anesthetized with pentobarbital (35 mg/kg body weight). Blood was obtained by puncturing the heart, and mixed with heparin (500 units/ml) or EDTA (3 mg/ml). Plasma was prepared from the heparinized and EDTA-anticoagulated blood samples by centrifugation at 1000 ×*g* for 10 min at 4 °C, and used for quantifications of LPLs, assay of lysoPLD activity and measurements of blood urea nitrogen, creatinine, urinary protein, adiponectin, leptin levels. In order to prevent artificial production of LPA by lysoPLD during plasma preparation, EDTA was used as an inhibitor of the lysoPLD as well as an anticoagulant. Urine was withdrawn by puncturing the bladder. These samples were stored at −80 °C until use.

2.3. Histological analysis

Kidneys and adipose tissue were quickly removed after rats were sacrificed, and then weighed. The tissues were fixed with 10% formaldehyde, embedded in paraffin, and cut at 5 μm. Pairs of serial sections were stained with either hematoxylin-eosin (HE) or Azan-Mallory (AZ). The areas observed were not restricted to narrow zones to avoid the possibility of missing highly localized lesions. To examine the entire specimen of each kidney sample, the observer scanned the slides at lower power for abnormalities and then evaluated their detail, extent, and distribution at higher powers. The parameters evaluated were 1) proximal tubular necrosis or degeneration, defined as coagulative necrosis or shrinkage with nuclear pyknosis of the proximal tubular epithelium, which was mainly seen in the tubules of the superficial and middle cortex, 2) tubular atrophy defined as thinning of the deep cortical tubular epithelial layer with a decrease in the amount of intraluminal eosinophilic secretion, 3) tubular regeneration defined as abnormal swelling of the tubular epithelial cells and nuclei, often with mitotic figures, which was mainly seen in the deep cortex, 4) interstitial fibrosis defined as deposition in the peritubular areas of blue-stained collagen fibers or abnormal thickening of the subepithelial basement membrane stained by AZ, while perivascular and peripelvic collagen bundles were disregarded, and 5) interstitial lymphocytic infiltrate, defined as peritubular collections of lymphocytes and plasma cells. Each parameter was scored as 0, absent; 1, only mildly present; 2, moderately present; and 3, either massively, universally, or severely present. The observer examined the histopathological changes without knowing the relationship between tissues and the experimental groups. To analyze the adipocyte area, 150 adipocytes per section were randomly selected and measured using Image J software from NIH.

2.4. Lipid extraction from plasma

Aliquots of plasma from EDTA-treated blood (0.4 ml) were diluted two-fold in saline with and without 20 mg KCl. Using the modified method of Bligh and Dyer, we extracted lipids from the plasma of EDTA-anticoagulated blood after adjusting the pH of the aqueous phase to 8–9 with 20% ammonium hydroxide, essentially as described previously [17].

2.5. LC-MS/MS of LPLs

LC-MS/MS was performed on a quadrupole-linear iontrap 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 [17].

2.6. Assay of choline-producing activity due to lysoPLD

The choline-producing activity of rat plasma diluted 10-fold was measured by enzyme-coupled fluorometric determination of choline released from 0.15 mM 16:0-LPC at 37 °C for 24 h as previously reported [9]. The lysoPLD activity is presented as nmol of choline/ml of undiluted plasma produced in 24 h.

2.7. Measurements of plasma urea nitrogen, creatinine and urinal protein

The concentrations of urea nitrogen and creatinine in plasma were determined using IatroLQ UN Rate (A) II (LSI Medience, Tokyo, Japan) that employed urease leucine dehydrogenase methods and Silicakid-S CRE (Kanto Chemical, Tokyo, Japan) that used creatinase – sarcosine oxidase peroxidase methods. Urinal protein was quantified by microTP-AR (Wako Pure Chemicals, Osaka, Japan) that employed pyrogallol red method.

2.8. Adiponectin and leptin determination

Adiponectin and leptin in rat plasma were determined using a high molecular weight adiponectin ELISA kit (Shibayagi Co., Gunma, Japan) and a leptin measurement kit (Morinaka Institute of Biological Sciences, Yokohama, Japan), according to manufacturer's instructions.

2.9. mRNA quantification

Total RNAs were extracted from kidney homogenates with RNAlater (Thermo Fisher Scientific, MA, USA) or NRK49F cells, using Sepasol RNA I Super G according to the manufacturer's manual. A Turbo DNA-free kit (Ambion, TX, USA) was used to remove contaminating DNAs from the RNAs obtained. The total RNA level was quantified by determination of ultraviolet absorbance at 260 nm, and its purity was verified by measuring the absorbance ratio at 260 and 280 nm. cDNA was prepared