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A metabolomic perspective of griseofulvin-induced liver injury in mice

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ABSTRACT

Griseofulvin (GSF) causes hepatic porphyria in mice, which mimics the liver injury associated with erythropoietic protoporphyria (EPP) in humans. The current study investigated the biochemical basis of GSF-induced liver injury in mice using a metabolimic approach. GSF treatment in mice resulted in significant accumulations of protoporphyrin IX (PPIX), *N*-methyl PPIX, bile acids, and glutathione (GSH) in the liver. Metabolomic analysis also revealed bioactivation pathways of GSF that contributed to the formation of GSF-PPIX, GSF-GSH and GSF-proline adducts. GSF-PPIX is the precursor of *N*-methyl PPIX. A six-fold increase of *N*-methyl PPIX was observed in the liver of mice after GSF treatment. *N*-methyl PPIX strongly inhibits ferrochelatase, the enzyme that converts PPIX to heme, and leads to PPIX accumulation. Excessive PPIX in the liver results in bile duct blockage and disturbs bile acid homeostasis. The accumulation of GSH in the liver was likely due to Nrf2-mediated upregulation of GSH synthesis. In summary, this study provides the biochemical basis of GSF-induced liver injury that can be used to understand the pathophysiology of EPP-associated liver injury in humans.

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1. Introduction

Griseofulvin (GSF) is used both in animals and humans for management of fungal infections. GSF is associated with multiple side effects including confusion, dizziness, nausea, diarrhea, and fatigue [1–3]. In addition, GSF is contraindicated to patients with porphyrias [4–6]. GSF causes hepatic porphyria in mice [7,8]. GSF upregulates the expression of the delta-aminolevulinate synthase (ALAS), the rate-limiting enzyme in heme synthesis pathway that increases the production of porphyrins [9]. Moreover, GSF treatment results in functional deficiency of ferrochelatase (FECH), the enzyme that converts protoporphyrin IX (PPIX) to heme [10]. Thus, GSF treatment in mice causes PPIX accumulation in the liver which leads to liver damage [7,8]. This phenotype is similar to human subjects with erythropoietic protoporphyria (EPP)-associated liver injury [7,11,12]. Therefore, GSF is commonly used as a tool drug to generate a mouse model for investigating EPP-associated liver injury [7,8].

Studies of GSF in mice have revealed hepatocellular injury, cholestasis, ductular proliferation and cirrhosis [8,13–15]. In addition, oxidative stress has been observed in the liver of mice treated with GSF [16]. Global gene analysis showed GSF-mediated alteration of genes that may be associated with inflammation, fibrosis, and cholestasis [17]. Nevertheless, the biochemical basis of GSF-induced liver injury remains understudied. The gaps are two-fold: (1) what are altered by GSF in liver metabolome; and (2) how does GSF cause the changes in liver metabolome? The current study addressed these two questions in mice using a metabolomic approach.

2. Materials and methods

2.1. Chemicals and reagents

Griseofulvin (GSF), protoporphyrin IX (PPIX), *N*-methyl PPIX, β-nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH) and L-proline (PRL) were purchased from Sigma–Aldrich (St. Louis, MO). Chemical standards of bile acids were obtained from Steraloids, Inc. (Newport, RI). All solvents for ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC–QTOFMS) analysis were of the highest grade commercially available.

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2.2. Animals and treatment

FVB/NJ mice (male, 8 weeks old) were fed with 2.5% GSF diet (w/w) or control diet ($n = 4$ per group) for 14 days. The dose and time of GSF treatment was chosen based on the previous studies that showed liver injury in mice [16,18]. On the last day of treatment, the mice were housed separately in metabolic cages to collect urine and feces. Afterwards, all mice were sacrificed and liver tissues were harvested. A section of liver tissues was fixed in 4% formaldehyde phosphate buffer. The remaining liver tissues were flash-frozen in liquid nitrogen and stored at −80 °C until further analysis. The study protocol was approved by the Institutional Animal Care and Use Committee.

2.3. Biochemical and pathological analysis

Biochemical analysis was conducted to measure alanine transaminase (ALT) and alkaline phosphatase (ALP) activities in serum. For pathological analysis, fixed liver tissues were subjected to dehydration in serial concentrations of alcohol and xylene followed by paraffin embedding. Four-micrometer sections of liver tissues were cut and stained with hematoxylin and eosin.

2.4. Sample preparation for metabolite analysis

Twenty five microliters of serum sample was mixed with 75 μL of methanol, followed by vortexing for 30 s and centrifugation at 15,000 × *g* for 10 min. Liver samples were homogenized in water (100 mg tissue in 400 μL water), and then a 200 μL aliquot of methanol was added to 100 μL of liver homogenate. The mixture was vortexed twice for 1 min and centrifuged at 15,000 × *g* for 20 min. Urine samples were prepared by mixing 50 μL of urine with 100 μL of acetonitrile and then centrifuged at 15,000 × *g* for 10 min. Feces were homogenized in water (1 mg of feces in 10 μL of H_2O), and then a 200 μL of acetonitrile: MeOH (1:1, v/v) was added to 200 μL of the resulting mixture, followed by centrifugation at 15,000 × *g* for 10 min. The supernatant was transferred to a new Eppendorf vial for a second centrifugation. One microliter of the supernatant was injected onto the UPLC–QTOFMS system for metabolite analysis.

2.5. In vitro metabolism of GSF

Incubations were conducted in 1× phosphate-buffered saline (PBS, pH 7.4), containing 10 μM GSF and 0.1 mg of mouse liver microsomes (XenoTech, LLC, Lenexa, KS) in a final volume of 190 μL. Incubations were performed in triplicates. After 5 min of pre-incubation at 37 °C, the reaction was initiated by the addition of 10 μL of 20 mM NADPH (final concentration 1.0 mM) and continued for 40 min with gentle shaking. GSH or PRL (final concentration 5.0 mM) was used to trap reactive metabolites. Incubations were terminated by adding 200 μL of ice-cold methanol, and then vortexed for 30 s and centrifuged at 15,000 × *g* for 10 min. One microliter of the supernatant was injected onto the UPLC–QTOFMS system for metabolite analysis.

2.6. UPLC–QTOFMS analysis

Chromatographic separation of metabolites was performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters Corporation, Milford, MA). The mobile phase A (MPA) was 0.1% formic acid in water, and the mobile phase B (MPB) was 0.1% formic acid in acetonitrile. The gradient began at 2% MPB and held for 1 min, followed by 12 min linear gradient to 95% MPB, held for 8 min, and then decreased to 2% MPB for column equilibration. The flow rate of mobile phase was 0.5 mL/min and the column

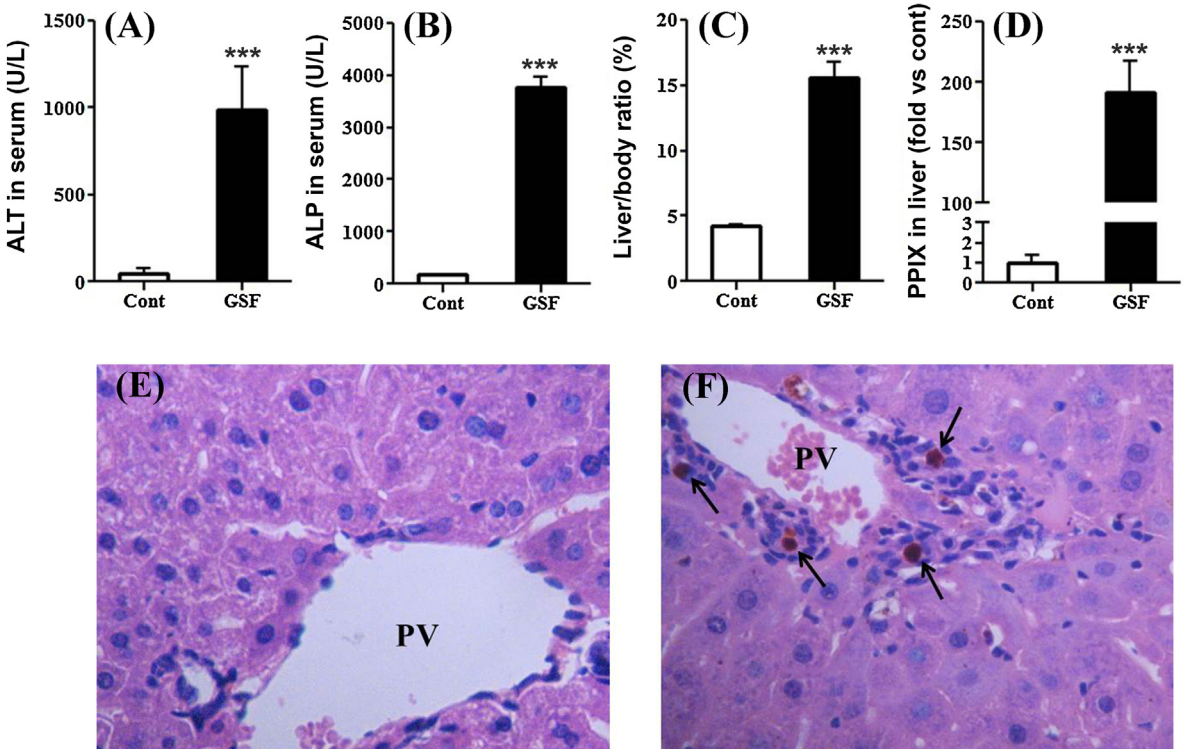

Fig. 1. GSF-induced liver injury in mice.
WT mice were treated with control diet or GSF diet for 14 days. (A and B) ALT and ALP activities in serum. (C) Liver to body weight ratios. (D) Relative quantification of PPIX levels in the liver. All data are expressed as means ± S.D. ($n = 4$). The data in control group were set as 1. ***$P < 0.005$ vs control. (E and F) Histological analysis of liver from control (E) and GSF group (F), H&E staining—200X. PV—portal vein. Arrows point to bile plugs.

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