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Furazolidone induces the activity of microsomal enzymes that metabolize furazolidone in chickens

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ABSTRACT

The nitrofuran antibacterial agent furazolidone (FZ) is still used in veterinary medicine in some countries in the Middle and Far East. The present study aimed to show the effect of FZ on the activity of microsomal enzymes that metabolize FZ, and to identify the enzyme that contributes to FZ metabolism in chickens. Wistar rats and White Leghorn chickens were administered FZ once a day for four consecutive days. FZ metabolism was accelerated by FZ administration in chickens, but not in rats. The elevation of FZ metabolism coincided with the induction of NADPH cytochrome P450 reductase (CPR) activity in chickens, but such induction was not observed in rats. FZ metabolizing activities were inhibited in the presence of a CPR inhibitor (diphenylene iodonium chloride) but not by the addition of archetypal cytochrome P450 inhibitors (CO or *n*-octylamine). The preset study concluded that FZ accelerated its own metabolism in the chicken by induction of the activity of CPR.

1. Introduction

The nitrofuran antimicrobial drug *N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone (furazolidone; FZ) has been used for >40 years for the treatment of certain bacterial and protozoal infections in humans and animals (e.g., broilers) [1]. The use of FZ in food-producing animals has been forbidden in European Union (EU) countries, the USA, Japan as well as many other countries owing to its mutagenic [2,25] and carcinogenic activities [3,4]. However, FZ is still in use in some countries of the Middle and Far East as a feed additive for livestock and shrimp [1,5,12–14,22] and also used for gastrointestinal infectious diseases in human [6,7]. In particular, domestic poultry is target animals for this antimicrobial drug.

Nitrofuran derivatives (including FZ) were reduced at the nitro group in the initial step of their biotransformation, and continued to undergo further reduction of the nitro group whereas other parts of these compounds might be metabolized in many different ways [16,21,28,29,31,32].

These derivatives (including FZ) are reduced in the alimentary tract and liver; this results in the formation of reduced metabolites that are reactive intermediates accompanying free-radical reactions that lead to formation of superoxide anion radicals and lipid peroxidation via redox cycling process [10,15–17,33]. It has been suggested that the nitro-reductive intermediates of nitrofuran derivatives are genotoxic [2,11,18,19]. The metabolite, which binds to protein covalently, is formed during the reductive metabolism of FZ in the livers of rats and pigs [8,9,20,21,24,26], as well as in the livers of turkeys and chickens [17,22]. It has also been suggested that the reductive metabolites of FZ bind to proteins as well as glutathione and react with DNA, consequently relating to the cytotoxicity that is exerted by several different mechanisms in Caco-2, HEp-2, and V79 cell lines [21]. FZ is therefore considered to have manifold bioactivities through initial nitro reduction and subsequent metabolic activations *in vivo*.

The metabolic rate of FZ might be facilitated by multiple doses of FZ in animals. Vroomen et al. [23] indicated that a decrease in the concentration of non-metabolized FZ in the urine of pigs was observed 2 days after FZ medication, and suggested that specific enzymes that metabolize FZ might have been induced. However, in chicken, one of main animal species to be treated with this drug, the effect of FZ on its metabolism little have been investigated.

This study showed the effects of FZ on its metabolic rate in chickens, and identified the main enzyme responsible for the increase of FZ metabolic rate in this species. This study revealed that FZ metabolic rate was increased by FZ treatment in chicken, and the fascilited activity of NADPH cytochrome P450 reductase (CPR) was responsible for the increase of the FZ metabolic rate.

2. Materials and methods

2.1. Chemicals

Nicotinamide adenine dinucleotide (NADPH), glucose-6-phospohate (G6P), and glucose-6-phosphate dehydrogenase (G6PDH)

Abbreviations: FZ, furazolidone; PB, phenobarbital; β-NF, β-naphthoflavone; APN, aminopyrine; APND, aminopyrine *N*-demethylase; DPI, diphenylene iodonium chloride; DMSO, dimethyl sulfoxide.

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were obtained from Oriental Yeast Company Limited (Tokyo, Japan). FZ was from Ueno Fine Chemical Industry Company Limited (Osaka, Japan), whereas *n*-octylamine, β-naphthoflavone (β-NF), and phenobarbital sodium (PB) were from Wako Pure Chemical Industries, Limited (Osaka, Japan). Nitrofurantoin, cytochrome c, and CPR were from Sigma–Aldrich (St. Louis, MO, USA), and diphenylene iodonium chloride (DPI) was from Dojin Chemical Laboratory (Kumamoto, Japan). All other chemicals were of analytical grade.

Before use, drugs were prepared as follows: FZ was suspended in 2% aqueous acacia solution, and PB dissolved in 0.9% saline solution. Acetone was dissolved in 0.9% saline for a 20% aqueous solution. β-NF (16 mg/ml) was dissolved in corn oil.

2.2. Animal treatment

Treatment of all animals was undertaken according to the policies of the International Animal Care and Use Committee of Hokkaido University (Sapporo, Japan).

Female Wistar rats (age, 6 weeks) were obtained from Japan SLC, Incorporated (Hamamatsu, Japan). They were housed in steel cages and fed a pellet diet (Nihon Nosan Kogyo Company, Yokohama, Japan) with water *ad libitum*. Female White Leghorn chickens (age, 2 months) were obtained from Hokkaido Central Chicken Farm (Hokkaido, Japan). They were housed in steel cages and fed a standard diet (Nihon Nosan Kogyo) and water *ad libitum*. Animals were maintained at 23 °C in a 12-h dark/light cycle (starting at 07:00 h).

Rats and chickens were divided into six groups of three. Each animal was administered FZ (high dose, 125 mg/kg/day; low dose, 62.5 mg/kg/day, by crop tube for 4 days), PB (80 mg/kg/day, intraperitoneally for 3 days), β-NF (80 mg/kg/day, intraperitoneally for 3 days), or acetone (5 mL/kg/day, by crop tube for 4 days) once a day. Control animals received 2% acacia solution (5 mL/kg/day, by crop tube, once a day, for 4 days).

2.3. Preparation of liver microsomal fractions

Rats and chickens were killed with carbon dioxide 24 h after the last dose and their livers removed. Liver microsomes were prepared according to the method of Omura and Sato [35]. Liver samples were homogenized with three volumes of ice-cold 1.15% KCl. The homogenate was centrifuged at 9000*g* at 4 °C for 20 min. The supernatant was centrifuged twice at 105,000 *g* at 4 °C for 70 min each. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4), frozen in liquid nitrogen, and stored at −80 °C until use. The microsome protein concentration was determined by the method of Lowry et al. [36].

2.4. Metabolism (disappearance) of furazolidone

The metabolism (rate of substrate disappearance) of FZ was measured according to modified methods of Sanwald et al. [37] and Yoshida and Kondo [38]. The reaction mixture consisted of 100 μM FZ (dissolved in dimethyl sulfoxide (DMSO)), 5 mM $MgCl_2$, 1 mM ethylenediamine tetra-acetic acid (EDTA), and 1 mg microsomal protein (or 2.5 μg of purified CPR in the inhibition study) in 1 mL of 0.1 M $K_2P_2O_7$ (pH 7.4) buffer. The mixture was pre-incubated for 10 min at 37 °C. The reaction was started by the addition of 10 μL G6PDH (200 U/mL) and 10 μL 50 mM NADPH mixture (in the case of CPR, 20 μL 50 mM NADPH). After 10 min, the reaction was terminated by the addition of 4 mL ethyl acetate. After the addition of 6.72 nmol nitrofurantoin to the mixture as an internal standard and mixing well for 1 min, the organic phase was separated by centrifugation at 1200*g* for 5 min. After evaporation of the organic phase, the residue was dissolved in the mobile phase. The sample was analyzed using a high-performance liquid chromatography (HPLC) system comprising a Shimadzu LC-6A pump, SPD-6A detector, and C-R6A recording data processor (Shimadzu Seisakusho Limited, Kyoto, Japan). HPLC was carried out using a Wakosil-2-5C18G column (150 mm × 4.6 mm I.D.; Wako), a mobile phase of acetonitrile:DW of 40:60, and a wavelength of 358 nm. In the inhibition study of FZ metabolism, *n*-octylamine dissolved in DMSO was added to the incubation mixture at a final concentration of 0.1 M as a CYP inhibitor, and DPI (in DMSO) was added to the incubation mixture at a final concentration of 0.2 M as a flavin enzyme inhibitor. Measurement of FZ metabolism are repeated three times.

2.5. CPR

The activity of CPR was assayed according to the method of Omura and Takesue [39]. The rate of cytochrome c reduction was followed at 415 nm using a spectrophotometer (Hitachi-U-300, Hitachi, Tokyo, Japan). Reductase activity was quantified using an extinction coefficient of 19.6 mM/cm. CPR dependent activity was independently repeated three times.

2.6. Effect of a CYP inhibitor on aminopyrine N-dimethylase (APND) activity

APND activity was determined by measuring the rate of formaldehyde formation according to the methods of Cooper and Brodie [40], and Nash [41]. The reaction mixture consisted of 1 mM aminopyrine, 4 mM $MgCl_2$, 10 mM G6P, and 1 mg microsomal protein in 1 mL of 0.1 M $K_2P_2O_7$ (pH 7.4) buffer. The mixture was pre-incubated for 10 min at 37 °C. The reaction was started by the addition of 10 μL G6PDH (200 U/mL) and 10 μL 50 mM NADPH mixture (in the case of CPR, 20 μL 50 mM NADPH). After 10 min, the reaction was terminated by the addition of 0.8 mL of 10% $ZnSO_4$. The rate of formaldehyde formation was followed by spectrophotometric means at 415 nm. In the inhibition study of APND, *n*-octylamine was added to the incubation mixture at a final concentration of 0.1 M.

2.7. Statistical analyses

Data are mean ± SD. Comparisons between two groups were carried out using the Student's *t*-test. Multiple comparisons were also undertaken using the Student's *t*-test, followed by adjustment of *p* values using the Bonferroni correction. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of FZ and CYP inducers on FZ metabolism

Fig. 1 shows the effects of administration of FZ or CYP inducers on FZ metabolism in the liver microsomes of rats and chickens. No significant change in FZ-metabolizing activity was observed in the FZ-treatment groups of the rat (Fig. 1A). PB- and acetone-treated rat groups showed slight (but significant) increases in FZ metabolism activity. In contrast to the effect of FZ treatment in rats, FZ administration to chickens resulted in large and significant increases in FZ-metabolizing activity, i.e., 2.1-fold in the 125 mg/kg FZ group and 1.6-fold in the 62.5 mg/kg FZ group, as compared with that of the control group (Fig. 1B). The chicken group administered acetone also showed a significant increase in FZ metabolism. However, a significant alteration of FZ metabolism was not observed in PB- and β-NF-treated chickens.