Elevated Warfarin Metabolism in Warfarin-Resistant Roof Rats (Rattus rattus) in Tokyo

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ABSTRACT:
Wild roof rats (Rattus rattus) live in proximity to human habitats, and they may carry numerous pathogens of infectious diseases. Pest control is important for public health, and warfarin is a commonly used rodenticide worldwide. However, continual use of warfarin may cause drug resistance in rodents and lead to failure of their control, especially in urbanized areas. In warfarin-resistant rats, the warfarin level in plasma was significantly lower after oral administration than that in the control warfarin-sensitive rats. Warfarin is metabolized by cytochrome P450 (P450), and hydroxylation of warfarin by P450 isoforms was significantly higher in warfarin-resistant rats (2-fold). Western blot analysis indicated that the level of CYP3A2 expression in warfarin-resistant rats was significantly larger than in warfarin-sensitive rats. The NADPH-P450 reductase activities in resistant rats were 8-fold higher than those in sensitive rats. In vivo, the administration of the P450 potent inhibitor proadifen (SKF-525A) increased the mortality of warfarin in the warfarin-resistant roof rats. We concluded that the mechanism of warfarin resistance in Tokyo roof rats is caused by increased clearance of warfarin.

Wild roof rats (Rattus rattus) live in proximity to human habitats, and they may carry numerous pathogens of infectious diseases, such as plague, hemorrhagic fever with renal syndrome, Weil’s disease, hepatitis E virus, and others. Although pest control is important for public health, continual use of coumarin-based rodenticides may cause drug resistance in rodents and lead to failure of their control, especially in urbanized areas.

Warfarin is a commonly used rodenticide worldwide. It inhibits coagulation of blood by inhibiting vitamin K epoxide reductase (VKOR) activity (Thijssen et al., 2004). An inadequate supply of vitamin K blocks the production of prothrombin and causes hemorrhaging.

Warfarin-resistant rats have already been reported in urban areas such as Liverpool, UK (Greaves et al., 1973), California and Texas (Jackson and Ashton, 1979), and Tokyo, Japan (Ito et al., 1981). Many studies have been performed to elucidate the mechanism of warfarin resistance. Rost et al. (2004) reported that warfarin resistance is attributable to a mutation (Tyr139Phe) in the vitamin K1 epoxide reductase complex subunit 1 (VKORC1) gene. Pelz et al. (2005) concluded that mutations in VKORC1 are the genetic basis of anticoagulant resistance of rodents in the experiments using recombinant VKORC1 mutants. Recently, Lasseur et al. (2006) reported that the resistant rats with mutation at position 139 in VKORC1 showed VKOR activity with reduced Vmax and very small Km, to the extent that Kcat (Vmax/Km) becomes larger than that of sensitive (wild-type) rats. This means that the resistant rat has higher VKOR activity than the sensitive rat at low concentrations of vitamin K epoxide under physiological conditions. However, mutations alone cannot explain all aspects of resistance in rodents (Wajih et al., 2004), and we actually could not detect the mutation at position 139 in the VKORC1 gene of Japanese roof rat. Pelz et al. (2005) agree that variations in anticoagulant uptake, metabolism, and clearance may influence the efficacy of rodenticides in vivo.

Another possible mechanism for animals to acquire warfarin resistance is by enhanced warfarin metabolism and elimination. Several forms of cytochrome P450 (P450) are known to metabolize warfarin (Daly and King, 2003). In rats, warfarin hydroxylation is mainly catalyzed by CYP2C, CYP2B, CYP1A, and CYP3A subfamilies (Fig. 1). The five hydroxides are identified as 4’-, 6-, 7-, 8-, and 10-hydroxywarfarin (Guengerich et al., 1982), and these primary metabolites are made more water-soluble by UDP-glucuronyltransferase activity and are excreted in urine.

In one study, Sugano et al. (2001) newly reported the potent resistance of wild roof rats to warfarin in the Tokyo area of Japan. They focused on the low level of mRNA expression of the warfarin-metabolizing enzyme, CYP3A, which catalyzes warfarin 10-hydroxylation in resistant rats, and concluded that reduced metabolic activation of warfarin might cause the resistance. However, it has been

ABBREVIATIONS: VKOR, vitamin K epoxide reductase; VKORC1, vitamin K1 epoxide reductase complex subunit 1; P450, cytochrome P450; HPLC, high-performance liquid chromatography; SKF-525A, proadifen; PAGE, polyacrylamide gel electrophoresis.
reported that CYP3A induced by St. John’s wort decreases the anti-coagulant effect and increases the tolerance for higher doses of warfarin in humans (Izzo, 2005). In the present study, we identified the clearance and metabolic profiles of warfarin in the Japanese roof rat to establish the novel mechanism of warfarin resistance in wild rodents.

Materials and Methods

Animals. Warfarin-sensitive and -resistant roof rats were supplied by Ikari Corporation (Chiba, Japan). Warfarin-resistant rats were caught in buildings in the Tokyo area, namely, Shinjuku (Tokyo), Akabane (Tokyo), Ichikawa (Chiba), and Kamagaya (Chiba), Japan. The rats were given food containing 0.025% warfarin for 1 month. Rats who survived more than 1 month were used as warfarin-resistant rats according to World Health Organization regulations. Warfarin-sensitive rats were originally from the Ogasawara Islands (Tanikawa and Suzuki, 1993). Six rats (three males and three females) were caught in the islands in 1989, and a closed colony is maintained in the laboratory of Ikari Corporation. Rats from the closed colony were used as warfarin-sensitive rats.

All rats were housed, one per cage, under standard laboratory conditions (12-h light/12-h dark) with food and water available ad libitum. They were used for experiments after 2 to 4 weeks of acclimatization. Treatment of all animals was performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University.

We determined the ages of roof rats by paired eye-lens weight (Tanikawa and Suzuki, 1993). Both eyes were removed and placed in 10% formalin immediately after sacrifice. We fixed eyes in formalin at room temperature for 4 weeks. After fixation, the tissue around the lens was removed, and the lenses were washed with distilled water. The lenses were dried at 60°C for 3 days in an oven, and were weighed to estimate the rat’s age according to the formula in a previous report (Tanikawa, 1993). Three- to six-month-old rats were used for all experiments.

Chemicals. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). β-Glucuronidase was obtained from Roche Diagnostics (Indianapolis, IN). Anti-rat CYP1A1, 2B1, 2C6, and 2C11 antibodies from goat and anti-rat CYP3A2 antibody from rabbit were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Anti-goat IgG and anti-rabbit IgG conjugated with horseradish peroxidase from rabbit were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Anti-rat CYP1A1, CYP2B1, CYP2C6, CYP2C11, and CYP3A2 antibodies were used to detect each P450 isoform. Anti-CYP1A1, -CYP2B1, and -CYP2C6 antibodies cross-react with CYP1A2, CYP2B2, and CYP2C13, respectively. Anti-rat CYP2C11 and CYP3A2 antibodies cross-react with other CYP2C and CYP3A subfamilies, respectively. The membrane was immunostained using diaminobenzidine. The results of staining were analyzed by using NIH Image v. 1.63 (Lennard, 1990).

Warfarin Metabolism. Warfarin metabolism by liver microsomes was analyzed by Kaminsky et al. (1979). MgCl2 (3 mM final concentration), glucose 6-phosphate (5 mM final concentration), and warfarin (400 μM final concentration) were mixed and added to microsomes (1 mg protein/ml final concentration) or potassium phosphate buffer for the negative control. The total volume of each sample was 1 ml. Samples were preincubated at 37°C for 5 min. A mixture of glucose-6-phosphate dehydrogenase (1 IU/ml final concentration) and NADPH (0.5 mM final concentration) was added to each sample to start the reaction. The reaction was kept at 37°C for 10 min, and 20 μl of 60% peric acid was added to stop the reaction. Samples were centrifuged at 3000g, 4°C for 5 min, and the supernatants were filtered through nitrocellulose (0.22 μm, 13 mm; Millipore Corporation, Billerica, MA). Filtered samples were injected into the HPLC system. We used an Inertsil ODS-2 column (GL Sciences Inc., Tokyo, Japan; 4.6 × 250 mm, 5 μm) with a detection wavelength of 306 or 307 nm. Mobile phase consisted of 1.5% acetic acid (pH 4.85)/acetoneitrile (70:30) or 15 mM KH2PO4 (pH 3.00, adjusted with 1 N HCl)/methanol/acetoneitrile (52:32:16).

Testosterone Metabolism. The incubation mixture for the assay of testosterone metabolism consisted of an NADPH generating system as the warfarin assay. Substrate concentration was 0.25 mM and corticosterone was spiked as an internal standard after the reaction. Testosterone metabolites were measured according to the methods of Ishizuka et al. (1998) by HPLC using a Supelcosil LC-318 (Supelco, Bellefonte, PA) column. Mobile phase consisted of methanol/water/tetrathydrofuran (55:55:10), and the detection wavelength was 245 nm.

NADPH-P450 Reductase. NADPH-P450 reductase activity was measured according to the method of Omura and Takesue (1970). Cytochrome c reduction was monitored at 550 nm and 37°C. The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.4, 50 μM cytochrome c, 50 μM NADPH, and 25 μg/ml microsomal protein. One unit of activity was defined as the amount of microsomal protein catalyzing the reduction of 1 nmol of cytochrome c/min under the above conditions.

VKORC1 Fragment cDNA Amplification and Sequencing. Total RNA was prepared from each liver sample of sensitive and resistant R. rattus (male) using TrizReagent (Sigma-Aldrich). The concentration and purity of the RNA fraction were determined spectrophotometrically at 260 and 280 nm, respec-
tively, oligo(dT)-primed total RNA was reverse-transcribed to cDNA using reverse transcriptase. VKORC1 primers were designed to amplify partial cDNA fragment, based on the conserved regions of rat, mouse, and human VKORC1 sequences retrievable from GenBank. The sense primer for VKORC1 was 5'-H11032-GTTTGCGGCTTGCACTATG-3'/H11032, and the antisense primer was 5'-H11032-ACCTCAGGGCTTTTTGACCT-3'/H11032. The polymerase chain reaction product was freshly cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), followed by the transforming of bacterium DH5α with plasmid DNA. The bacterial cells were cultured for 14 h on average. Purified plasmids were directly sequenced by the method of dye terminator cycle sequencing. The cycle sequencing reactions were performed with the vector-specific M13 primers (Invitrogen) using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The nucleotide sequence of the amplified fragment was determined by an automated DNA sequencer (ABI Prism 310 Genetic Analyzer).

**Statistical Analysis.** Student's *t*-test and two-way analysis of variance were performed using JMP IN v. 5.1. *p* < 0.05 was considered significant. Columns show the means, and error bars show the S.D. in bar charts.

**Results**

**Warfarin Concentration in Plasma in Roof Rats.** Warfarin concentrations were determined after oral administration to investigate warfarin concentration of warfarin-sensitive and -resistant rats (Fig. 2). The warfarin concentration in the plasma of warfarin-sensitive rats was higher than that of warfarin-resistant rats at 60 min after administration (8-fold).

**Warfarin-Metabolizing Enzymes in Roof Rats.** We also measured the warfarin metabolic activities in liver microsomes of roof rats. The rates of formations of all hydroxywarfarins were significantly higher in warfarin-resistant rats than those in -sensitive rats after the reaction, resulting in a 2-fold difference in total warfarin-metabolizing activities (Fig. 3).

To determine the expression of P450s, we compared expressions of each P450 related to warfarin metabolism (Fig. 4). Expression of CYP3A subfamily was significantly higher (4.1-fold) in warfarin-resistant rats than -sensitive rats. In male rats, expression of CYP2C11 was also significantly (1.2-fold) higher in warfarin-resistant rats than that in -sensitive rats. Expressions of the other P450s (CYP1A, CYP2B, and CYP2C6) showed no significant difference between warfarin-resistant and -sensitive rats. It should be noted that despite the fact that all the pathways of warfarin metabolism showed higher activities in resistant rats, not all the P450 isozymes catalyzing those pathways showed higher expression levels.

We also measured P450 contents (Omura and Sato, 1964). Although the contents of P450 showed the tendency of induction in resistant male rats, the induction was not significant (data not shown).

**Testosterone Metabolism.** Since the expression of CYP3A was induced in the liver of resistant rat, we measured CYP3A-dependent activity using testosterone as a model substrate. The testosterone 6β-hydroxylation activity was significantly elevated in the liver of
resistant rat; metabolic activity was 4-fold higher in resistant than in sensitive rats (Fig. 5). The 16β- and 7α-hydroxylase activities were also higher in livers of resistant rats than in those of sensitive animals (1.9- and 1.8-fold, respectively). There was no significant difference in the activity of 2α-hydroxylation, which was catalyzed by CYP2C11, between resistant and sensitive animals (data not shown).

**NADPH P450 Reductase Activity.** Since the rate of P450 reduction is a significant determinant of the rate of P450-dependent reactions, we measured the NADPH-P450 reductase-dependent activities in sensitive and resistant rats (Fig. 6). The activity of NADPH-P450 reductase-dependent cytochrome c reductase in liver microsomes was 3.00 ± 1.77 nmol/min/mg in sensitive rats, and 23.42 ± 3.91 nmol/min/mg in resistant animals.

**In Vivo Inhibition of Warfarin Metabolism in Resistant Rat.** We treated warfarin-resistant rats with warfarin or the P450 potent inhibitor, SKF-525A (Table 1). All rats that were administered warfarin or SKF-525A alone survived during the administration period (12 days), and we did not observe any hemorrhage symptom among them. However, in the cotreated group, which was administered 0.05% SKS-525A and 0.025% warfarin, the number of dead was one of four rats at day 12, and yet another rat died at day 14. We observed that two of four male rats died after cotreatments of 0.1% SKF-525A and 0.025% warfarin at day 12.

**Discussion.**

A recent study showed that substituted VKORC1 (Tyr139Phe) was poorly inhibited by warfarin, and the mutation in VKORC1 was one of the factors for warfarin resistance (Lasseur et al., 2006). Thus, we cloned and sequenced (data not shown) the fragments of the VKORC1 gene from liver of sensitive and resistant R. rattus. However, in this study, we did not detect the mutation at position 139 in VKORC1 in liver of resistant roof rat. Then, we focused on the possibility that the clearance of warfarin in resistant rat might be accelerated, since P450 metabolism has been suggested to cause warfarin resistance in humans. Also, induction of P450 by other drugs decreases warfarin levels in the human (Dingemanse and van Giersbergen, 2004).

We suggest that elevation of the ability to detoxify foreign chemicals due to the induction of enzyme expression or genetic polymorphism in wild roof rats may adapt them to a rodenticide-contaminated environment. Exposure to environmental pollutants such as pesticides might induce the expression of P450 isoforms in wild rodents to accelerate detoxification. In fact, Fujita et al. (2001) reported the elevation of CYP2B and CYP3A expression in wild voles (Clethrionomys rufocanus) that inhabited highly pesticide-contaminated areas. Alternatively, polymorphically higher expression of P450 may enhance the ability of detoxification. Hoshi et al. (1995) also found a polymorphic P450 isofrom in wild rodents, and reported the extensive and poor metabolizers of xenobiotics.

We studied the warfarin residue level in plasma of sensitive and resistant roof rats after oral administration (Fig. 2). Warfarin levels in plasma were lower in warfarin-resistant than in -sensitive animals. Warfarin metabolic activities in resistant rats were higher than those of sensitive animals, as we expected (Fig. 3).

According to the immunoblotting results (Fig. 4), high expression of CYP3A2 seems to be the reason for high warfarin metabolism. Sugano et al. (2001) showed suppression of CYP3A2 mRNA expression in their warfarin-resistant rat. In the current study, we measured the expression levels of CYP3A2 apoprotein in addition to enzymatic reaction. Moreover, the hydroxylization activity of testosterone 6β, which was catalyzed by CYP3A2 in male rat, was also higher in resistant rat compared with sensitive animals (data not shown).

Expression of the CYP3A subfamily has been demonstrated to be regulated by nuclear receptors, such as pregnane X receptor and glucocorticoid receptor, and neonatal hormonal imprinting (Pascussi et al., 2000). In rats, CYP3A2 has a gender difference in its expression profile, and the concentration of this isoform is usually low in immature males. Although Sugano et al. (2001) did not indicate the ages of their rats, an estimation of age might be essential to consider the P450 expression in wild rats.

In our study, the formation of 10-hydroxywarfarin, formed by a CYP3A-dependent reaction, was higher in warfarin-resistant rats than in -sensitive rats. However, formation of other metabolites (4'-, 6-, and 8-hydroxywarfarins) was also higher in resistant rats. Assay of testosterone metabolism also elucidated the acceleration of total P450-dependent metabolisms in resistant rats. We found not only the elevation of CYP3A-dependent 6β-testosterone hydroxylation in resistant rats, but also a high level of 7α- and 16β-hydroxylase activities.
(CYP2A- and CYP2B-dependent activities, respectively). Thus, high CYP3A might not be the only reason for higher warfarin metabolism in resistant rats. NADPH-P450 reductase activity is involved in every P450 reaction, and it might be the rate-determining step of reactions of P450. We found that NADPH-cytochrome c reductase activity, dependent on NADPH-cytochrome P450 reductase, was markedly higher in resistant rats than in sensitive rats. This may be the reason for high activities of every pathway of warfarin metabolism in resistant rats. To establish the P450 contribution to warfarin resistance, we treated roof rats with feeding bait containing warfarin and the P450 potent inhibitor SKF-525A (Table 1). Although the number of rats used for this examination was limited, we have already confirmed that warfarin injection was not fatal for the resistant rats, and the mortality rate was 0%. Increase of mortality rate in rats cotreated with warfarin and SKF-525A indicated the possibility that P450-dependent warfarin metabolism might contribute to warfarin resistance in wild rodents.

In the present study, we reported that warfarin-resistant rats had high metabolic activity of warfarin by P450, which might be due to the high expression of CYP3A and elevated activity of NADPH-P450 reductase. We concluded that the elevation of P450-dependent warfarin metabolism also gave rise to warfarin resistance in wild rodents. This is the first report that showed evidence of P450-dependent metabolisms caused the pesticide resistance in wild mammals.

References

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