A Novel Mutation in VKORC1 and Its Effect on Enzymatic Activity in Japanese Warfarin-Resistant Rats

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(Received 13 April 2012/Accepted 8 September 2012/Published online in J-STAGE 26 September 2012)

ABSTRACT. Warfarin is a rodenticide commonly used worldwide. It inhibits coagulation of blood by inhibiting vitamin K 2,3-epoxide reductase (VKOR) activity. An inadequate supply of vitamin K blocks the production of prothrombin and causes hemorrhage. Recently, warfarin-resistant brown rats (Rattus norvegicus) were found around the Aomori area of Japan. There is no significant difference in the metabolic activity of warfarin in sensitive and resistant brown rats. To clarify the mechanism underlying warfarin resistance, we cloned the VKORC1 gene from rats and identified a novel substitution of arginine to proline at position 33 of the VKORC1 amino acid sequence. Then, we determined the differences in kinetics of VKOR activity between warfarin-resistant and sensitive rats. Hepatic microsomal VKOR-dependent activity was measured over a range of vitamin K epoxide concentrations from 6.25 to 150 μM. The V_max values of resistant rats (0.0029 ± 0.020 nmol/min/mg) were about one tenth of those of sensitive rats (0.29 ± 0.12 nmol/min/mg). The K_m values of resistant rats (47 ± 32 μM) were similar to those of sensitive rats (59 ± 18 μM). Warfarin-sensitive rats exhibited enzyme efficiencies (V_max/K_m) which were ten-fold greater than those observed in resistant rats. It may mean that VKOR activity of warfarin-resistant Aomori rats is almost lost, because their enzymatic efficiencies are very low even without warfarin. Further studies are needed to clarify how these rats can survive with a markedly reduced VKOR activity and how they simultaneously exhibit warfarin resistance.

KEY WORDS: anticoagulant, kinetic parameter, Rattus norvegicus, VKOR, warfarin resistance.


Brown rats (Rattus norvegicus) can carry numerous pathogenic organisms, such as those causing plague and hemorrhagic fever with renal syndrome. Therefore, pest control is one of the key priorities for public health. Coumarin-derived rodenticides such as warfarin are commonly used to control rat populations worldwide.

The pharmacological target of warfarin is vitamin K 2,3-epoxide reductase (VKOR). Through the inhibition of VKOR, warfarin blocks the vitamin K cycle and inhibits the γ-carboxylation of vitamin K-dependent blood-clotting factors II, VII, IX, and X. Because these clotting factors are not activated following the inhibition of VKOR activity, warfarin causes lethal hemorrhage [2, 18].

Warfarin has been widely used since the 1950s to control rodent populations. The repeated use of warfarin may cause drug resistance in rodents, and lead to a failure to control them. Resistance to warfarin was first observed in Scotland in 1958 [1]. Since then, there have been many reports of resistant rats all over the world including Great Britain [1], Denmark [9], Germany [17] and the U.S.A. [5]. Several mechanisms of warfarin resistance in wild rats have been suggested, including the possibility of mutation of the VKOR gene. Rost et al. reported that warfarin resistance in rats was attributable to the 139 tyrosine substitution to phenylalanine in the VKORC1 gene [13]. Pelz et al. reported that the 139 tyrosine substitution to phenylalanine caused the greatest level of insensitivity of VKOR to warfarin when this mutated enzyme was expressed in HEK 293 cells [11]. More recently, an alternative mechanism of warfarin resistance has been suggested which involves the acceleration of warfarin excretion by the cytochrome P450 system in resistant black rats in Japan [3].

Meanwhile, in Asian countries, there have been few reports of warfarin-resistant rats. In Japan, wild rat populations are dominated by black rats in rural and urban areas. In the 1980s, resistance in black rats was reported in the Tokyo region [4]. However, the appearance of resistance in brown rats is very recent in Japan and was first reported in Aomori in 2006 [16]. In this paper, we investigated the structure and enzymatic properties of VKOR from sensitive and resistant Japanese brown rats in order to compare the mechanism of warfarin resistance to that of wild rats in Europe.

MATERIALS AND METHODS

Chemicals: HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan). CuSO4·5H2O, vitamin E and vitamin K were from Kanto Chemicals (Tokyo, Japan). Bovine serum albumin, dicoumarol, diethyl ether and warfarin were from Sigma Aldrich Inc. (St. Louis, MO, U.S.A.)
Dithiothreitol (DTT), dichloromethane, ethanol, methanol, H₂O₂, K₂HPO₄, KH₂PO₄, MgCl₂, Na₂CO₃, NaCl, NaOH, perchloric acid and phenol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Warfarin metabolites (4’-, 6’-, 7-, 8- and 10-hydroxywarfarin) were obtained from Ultrafine Chemicals (Manchester, U.K.).

Animals: Warfarin-resistant brown rats (16 months old) were supplied by the Ikari Corporation (Chiba, Japan). Originally, warfarin-resistant male and female brown rats, were maintained in the laboratory of the Ikari Corporation. They were given food containing 0.005% warfarin for 12 days, and the rats that survived more than one month were identified as warfarin-resistant rats. Sprague-Dawley rats (13–16 months old) were used as warfarin-sensitive rats. All rats were housed, one per cage, under standard laboratory conditions (with a 12 hr light/12 hr dark cycle) with food and water available ad libitum. After the administration of warfarin and one month of acclimatization, they were used for experiments. Treatment of all animals was performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University.

Preparation of liver microsomes: Livers were harvested from warfarin-sensitive and resistant rats after sacrifice using sevoflurane excessive inhalation, and liver microsomes were prepared according to the method of Omura and Sato [10]. The livers were homogenized with 3 times their volume of potassium phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 9,000 g at 4°C for 20 min. The supernatants were decanted to an ultracentrifugation tube and centrifuged at 105,000 g at 4°C for 60 min. Pellets were homogenized in potassium phosphate buffer (0.1 M, pH 7.4) on ice. The microsomal homogenates were transferred to 1.5 ml tubes and stored at −80°C until use.

Determination of VKORC1 mRNA expression levels: Total RNA was prepared from each liver sample of warfarin-sensitive and resistant rats using TriReagent (Sigma-Aldrich). RNA concentrations and purity were determined spectrophotometrically at 260 and 280 nm respectively. The cDNA was synthesized from total RNA. A mixture of total RNA, oligo(dT) (TOYOBO, 0.25 pmol/µl, final concentration) and DDW (mess up to 5 µl) was incubated at 70°C for 10 min and then cooled on ice for 1 min. After that, 4 µl of 5-fold concentrated RT buffer, 10 µl of 2.0 mM dNTP mixture and 1 µl of reverse transcriptase (ReverTra Ace, TOYOBO) were added to the mixture and then incubated at 42°C for 50 min and at 99°C for 5 min. The cDNA was amplified by PCR using specific primers for rat VKORC1. The sequences of the sense and antisense primers were 5’-GTGTCCTGGCCTG-TACGGTGCACATC-3’ and 5’-TAAGGCAAAGCT- CATGTCAGCCTGG-3’, respectively. PCR was performed by using Ex Taq under the following conditions: 94°C for 90 sec, 35 cycles of 94°C for 30 sec, 63°C for 45 sec, 72°C for 60 sec and final extension at 72°C for 5 min. The PCR products were used for sequencing analysis directly. Sequencing PCR reactions were performed at 96°C for 7 min, 40 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, using a BigDye Terminator version 1.1 (Applies Biosystems). The sense and antisense primers for cDNA sequencing were 5’-TGTCGACATGGCACCACCTGGAG-3’ and 5’-ATGGAGTTGGAGCTCCAGGCTTGGT-3’. Ethanol precipitation was performed after the amplification reaction and the nucleotide sequence was analyzed by an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer) following the manufacturer’s instructions.

Preparation of vitamin K epoxide: Vitamin K epoxide was prepared according to the method of Tishler et al. [19]. First, 50 µl of 30% H₂O₂ and 250 µl of DDW containing 0.1 g Na₂CO₃ were added to a solution of 0.1 g vitamin K in 5 ml of ethanol. The mixture was kept at 75°C and stirred vigorously for about 15 min, until the solution changed color from yellow to pale pink. The mixture was cooled for 5 min, diluted with 9 ml of DDW, and extracted with 70 ml of diethyl ether. After centrifugation (1,000 g, 10 min), the diethyl ether layer was isolated and evaporated by a centrifugal evaporator (EYELA, Tokyo, Japan). The reaction product was refined by HPLC [Pump: PU-980 (JASCO), column: Inertsil PREP-ODS column, 30.0 × 250 mm (GL Science Inc.), guard column: Mighysil, RP-18 GP Aqua (Kanto), 4.6 × 5 mm, 5 µm, detector: SPD-6AV (SHIMADZU), wavelength: 270 nm, flow rate: 7.0 ml/min, mobile phase: methanol containing 3% DDW]/[1120 Compact LC (Agilent), column: TSKgel ODS-120T, 2.0 mmI.D. ×250 mm (TOSOH) guard column: POLAR-RP, 4 × 2.0 mm (Phenomenex), wavelength: 270 nm, Flow rate: 0.4 ml/min, mobile phase: methanol contain-
ing 4% DDW]. The concentration of vitamin K epoxide was determined spectrophotometrically using molar absorption coefficients of 30,800 M⁻¹ cm⁻¹ at 266 nm (spectrophotometer: U-3300 HITACHI) [20]. Vitamin K epoxide was stored at 4°C and shielded from light until use.

**Enzymatic reaction for kinetic studies of vitamin K epoxide reductase activity**: VKOR activity was assayed using rat microsomes [3]. Microsomes from warfarin-sensitive and resistant rats were diluted in 0.1 M HEPES buffer (pH 7.4) to a final protein concentration of 0.4 mg/ml. The reaction mixture (500 µl total volume) contained 6.25, 12.5, 25, 50, 75, 100 or 150 µM vitamin K epoxide. Samples were pre-incubated at 37°C for 5 min, and the reaction was initiated by the addition of 2 mM DTT solution in 0.1 M HEPES buffer. The incubation time was 5 min, and the reaction was stopped by addition of 500 µl iced dichloromethane. To wash the solution, 0.5 ml 1.5% NaCl solution was added, and then 3.7 ml dichloromethane containing vitamin E as internal standard was added. After centrifugation (1,000 g, 10 min), the aqueous layer was removed by an aspirator. For evaporation, 3 ml of the dichloromethane layer was isolated and evaporated by a centrifugal evaporator. Vitamin K concentration was measured by HPLC [Pump: PU-980 (JASCO), detector: SPD-6AV (SHIMADZU), column: LiChrospher RP-18(e), 5 µm, 10 cm (MERCK), guard column: Mighysil, RP18GP Aqua (Kanto), 4.6 × 5 mm, 5 µm, wavelength: 270 nm, flow rate: 1.0 ml/min, mobile phase: methanol containing 3% DDW)]. The VKOR activity curves were fitted using nonlinear regression and the Michaelis-Menten equation. Estimations of apparent $K_m$ and $V_{max}$ were obtained by graphical analysis of Hanes-Woolf plots using Hyper ver. 1.01 (http://wvlc.uwaterloo.ca/biology447/modules/module7/hyper/index.htm). The data were also analyzed by fitting a hyperbola using Graph Pad Prism (GraphPad Software, Inc., San Diego, CA, U.S.A.).

**Statistical analysis**: After confirming the homoscedasticity by F-test, Student’s $t$-tests were performed using JMP IN v.5.1. (SAS); results were considered to be significant at the $P<0.05$ level.

### RESULTS

**Determination of VKORC1 mRNA expression levels**: VKORC1 mRNA expression levels in the livers of 6 sensitive and 6 resistant brown rats (3 male and 3 female in each case) were measured using real-time PCR. Figure 1 indicates that there was no significant difference in the mRNA expression levels of VKORC1 between sensitive and resistant rats.

**Sequencing analysis of VKORC1**: Figure 2 shows the cDNA sequences of VKORC1 and the deduced amino acid sequences. The base substitution at 99 (G→C) led to a mutation of the amino acid at position 33 from Arg to Pro. This substituted amino acid was detected in all warfarin-resistant rats (n=7). Amino acid substitution at 139 has been reported to cause warfarin resistance [11]; however, we did not detect a mutation at position 139 in the VKORC1 gene of Aomori rats.

**Kinetic parameters of VKOR activity**: Kinetic parameters were estimated by measuring the velocity of the VKOR enzymatic reaction. Figure 3 shows typical results fitted by nonlinear regression using the Michaelis-Menten equation. Sensitive rats showed higher $V_{max}$ and similar $K_m$ values than resistant rats ($K_m$ sensitive rats: 67 ± 39 µM, resistant rats: 41 ± 35 µM, $V_{max}$ sensitive rats: 0.32 ± 0.19 nmol/min/mg protein, resistant rats: 0.033 ± 0.024 nmol/min/mg protein). Therefore, warfarin-sensitive rats showed a five-fold higher $V_{max}/K_m$ value compared to warfarin-resistant rats (sensitive rats: 0.0048 ± 0.00066, resistant rats: 0.00096 ± 0.00029). There is significant difference in $V_{max}/K_m$ values between sensitive and resistant rats. To investigate the effect of warfarin, we then performed the same assay using 0.1 µM warfarin and found that no activity was detected.

### DISCUSSION

Warfarin-resistant black rats (*Rattus rattus*), known as “super rats”, have been reported in Tokyo, Japan since 1981 [4]. Uncontrolled increases in the population of these rats are not only a public health problem as they carry zoonotic...
pathogens, they are also a nuisance to society as they often gnaw electric cables and damage civil infrastructure. Meanwhile, warfarin-resistant brown rats (*Rattus norvegicus*) were only recently reported in Aomori, Japan in 2006. Like black rats, brown rats are also a social problem, and rodenticides have been used to control their populations. In this study, we investigated the VKOR from sensitive and resistant Japanese brown rats in order to compare the mechanism of warfarin resistance to that of wild rats in Europe.

Previous studies in warfarin-resistant black rats have indicated that the rate of CYP-dependent warfarin metabolism was higher in these rats, and this enhanced clearance of warfarin might be one of the major reasons why black rats in Tokyo possessed warfarin resistance [3]. Thus, we compared hepatic microsomal warfarin hydroxylase activities in warfarin-sensitive and resistant brown rats (data not shown). However, we did not find any significant differences in the activity of warfarin hydroxylation between sensitive and resistant rats.

We then focused on the structural and functional differences in the VKORC1 gene between warfarin-sensitive and resistant rats. For the former, we measured the expression levels of VKORC1 mRNA in sensitive and resistant rats. As indicated in Fig. 1, we could not detect any differences in the expression levels of this molecule between sensitive and resistant rats.

Mutations in VKORC1 may change the activity of VKOR. Rost et al. reported that HEK293 cells which overexpressed mutated VKORC1 (Tyr139Cys) had lower VKOR activity than that of wild-type cells, even in the absence of warfarin [12]. However, activity of the mutant VKOR was not inhibited by warfarin. Pelz et al. suggested that Tyr139 might be a part of the warfarin binding site in VKORC1 [11]. Lasseur et al. reported that warfarin resistance in brown rats in France was attributable to a mutation (Tyr139Phe) in VKORC1 [6]. The mutation at position 139, which is reported to be a warfarin binding site, caused reduced VKOR activity (low *V*<sub>max</sub>) in warfarin-resistant brown rats. However, a very low *K*<sub>m</sub> value for VKOR activity in these resistant rats led to equal or even higher enzymatic efficiency (*V*<sub>max</sub>*K*<sub>m</sub>) compared with sensitive rats. Thus, in this study, we cloned the VKORC1 gene from Aomori rats and sequenced it to investigate the possibility that these rats possessed a Tyr139Cys mutation, which might contribute to warfarin resistance. As shown in Fig. 2, we found a novel mutation at position 33 in resistant Aomori rats, and the position of the amino acid substitution in the Aomori brown rats is different from that of the warfarin-resistant rats from France.

We then measured VKOR-dependent activity in the liver microsomes of sensitive and resistant rats. Warfarin-sensitive rats showed a five-fold higher *V*<sub>max</sub>*K*<sub>m</sub> value than was seen in warfarin-resistant rats (Table 1). VKOR activity was long believed to be carried out by a large multienzyme complex, but it is currently suggested to be the small, single protein, VKORC1 [7, 13]. So, we regarded alteration of this enzymatic activity was resulted by the effect of amino acid substitution of VKORC1. And, these results indicate that even at low substrate concentrations (i.e., at biological concentrations), mutant VKORC1 shows low enzymatic efficiency. That is to say, it is expected that VKORC1 activity would be considerably lower or almost lost in warfarin-resistant rats. Spohn et al. reported that VKORC1-deficient mice developed normally until birth, and then died within 2–20 days postpartum due to extensive, predominantly intracerebral hemorrhage [15]. In a further study, we need to confirm the relationship between this mutation and its effect on enzymatic activity.

We also measured VKORC1 activity in the presence of a very low concentration of warfarin (0.1 µM), and found that the activity was beneath the detection limit. This might mean the inhibition constant was similar to or lower than that of sensitive rats. The base substitution at position 33 of VKORC1 had a completely different effect on enzymatic activity compared to the substitution at position 139 in French rats.

**Table 1. Kinetic parameters of vitamin K epoxide reductase in warfarin-sensitive and resistant brown rats**

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<th><em>V</em>&lt;sub&gt;max&lt;/sub&gt; [nmol/min/mg protein]</th>
<th><em>K</em>&lt;sub&gt;m&lt;/sub&gt; [µM]</th>
<th><em>V</em>&lt;sub&gt;max&lt;/sub&gt;<em>K</em>&lt;sub&gt;m&lt;/sub&gt;</th>
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<tr>
<td>Sensitive (n=3)</td>
<td>0.32 ± 0.19</td>
<td>67 ± 39</td>
<td>0.0048 ± 0.00066</td>
</tr>
<tr>
<td>Resistant (n=3)</td>
<td>0.033 ± 0.024</td>
<td>41 ± 35</td>
<td>0.00096 ± 0.00029*</td>
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Warfarin-sensitive rats showed five-fold higher *V*<sub>max</sub>*K*<sub>m</sub> values than did warfarin-resistant rats when the VKOR-dependent activity in liver microsomes of sensitive and resistant rats was measured. *: *P*<0.05.
To summarize the present study, we identified a novel mutation in VKORC1 in Japanese warfarin-resistant brown rats. These mutants showed lower $V_{max}$ and similar $K_m$ values for vitamin K epoxide reduction compared to those of sensitive rats, indicating that the activity at a low substrate concentration is lower compared to that of wild type rats. However, the mechanism by which Aomori brown rats compensate for reduced VKOR activity remains unclear. We performed homology modeling and a docking simulation for VKORC1 and VKO, and found that the difference between sensitive rats and resistant rats was only very slight (data not shown).

After the crystal structure of the fusion protein formed from VKOR and a Trx-like domain derived from Synechococcus sp. was determined, an electron transfer pathway was proposed to describe how electrons flow from reduced cysteines in newly synthesized proteins in the endoplasmic reticulum lumen through a Trx-like protein and VKOR to reduce either vitamin K epoxide to quinine, or quinine to hydroquinone [8, 14]. In mammalian VKOR, most mutations are mapped close to the binding site of quinone, the striking clustering of the mutations indicating that warfarin binds to the same site as quinone or at least close to it. The position of the 33rd amino acid in VKORC1, which is substituted in the warfarin-resistant rats of this study, is also close to the binding site of quinine. Further studies are needed to clarify this point.

Additionally, the mechanism of compensation for reduced VKOR activity in Aomori brown rats is still unclear. Several possibilities may be suggested. There may exist another electron transport pathway which is not the target of warfarin instead of vitamin K epoxide reductase. Alternatively, these warfarin resistant rats may obtain high vitamin K producing enterobacteria, and γ-carboxylation can be operative despite the vitamin K is not regenerated cyclically. Another possibility is the existence of unknown γ-carboxylation activation system independent of vitamin K cycle. A further study is needed to clarify how these rats survive with a markedly reduced VKOR activity and acquired warfarin resistance at the same time.

REFERENCES


