Warfarin Resistance in a French Strain of Rats

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ABSTRACT: A warfarin-resistant strain and a warfarin-susceptible strain of wild rats (Rattus norvegicus) maintained in enclosures of the National Veterinary School of Lyon (France) were studied to determine the mechanism of the resistance to anticoagulant rodenticides. A low vitamin K epoxide reductase (VKOR) activity has been reported for many resistant rat strains. As recently suggested, mutations in the vitamin K epoxide reductase subunit 1 (VKORC1) gene are the genetic basis of anticoagulant resistance in wild populations of rats from various locations in Europe. Here we report, for our strain, one of the seven described mutations (Tyr139Phe) for VKORC1 in rats. In addition, a low expression of mRNA encoding VKORC1 gene is observed in resistant rats, which could explain their low VKOR activity. We calculated kinetic parameters of VKOR in the warfarin-resistant and warfarin-susceptible rats. The $V_{\text{max}}$ and the $K_m$ of the VKOR obtained in resistant rats were lowered by 57 and 77%, respectively, compared to those obtained in susceptible rats. As a consequence, the enzymatic efficiency ($V_{\text{max}}/K_m$) of the VKOR was similar between resistant and susceptible rats. This result could be a good explanation to the observation that no clinical signs of vitamin K deficiency was observed in the warfarin-resistant strain, while a low VKOR activity was found. VKOR activity in warfarin-resistant rats was poorly inhibited by warfarin ($K_i$ for warfarin is 29 $\mu$M and 0.72 $\mu$M for resistant and susceptible rats, respectively). © 2005 Wiley Periodicals, Inc. J Biochem Mol Toxicol 19:379–385, 2005; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20104

KEYWORDS: Rattus norvegicus; Anticoagulant; Resistance; VKOR; VKORC1; Kinetic parameters

INTRODUCTION

Control of Rattus norvegicus populations is performed with rodenticides and mainly with antivitamin K (AVK) belonging to coumarin derivatives. AVK compounds are inhibitors of the vitamin K cycle and so prevent the $\gamma$-carboxylation of vitamin K dependent clotting factors II, VII, IX, and X. Because the vitamin K cycle is inhibited [1,2] and consequently the clotting factors are not activated, AVK causes lethal bleeding. First generation AVK compounds, such as warfarin, have been widely used since 1950s. Resistance to warfarin was first observed in Scotland in 1958 [3]. Since then, many resistant rat strains have been identified (in Great Britain [3], Denmark [4], Germany [5], USA [6], and Scotland [7]). It has been established that resistance to warfarin in rats is due to a single autosomal gene $Rw$ (Resistance to Warfarin [8]) located on chromosome 1 [9]. This $Rw$ gene is linked to the microsatellite D1Rat219 [10].

The pharmacological target of warfarin is the vitamin K epoxide reductase (VKOR). Warfarin is a potent inhibitor of VKOR in susceptible rats. On the contrary, warfarin is a weak or no-inhibitor of the VKOR activity in most resistant rat strains. Moreover, VKOR is not only weakly inhibited by warfarin in resistant rats, but surprisingly basal VKOR activity is also frequently lowered in warfarin-resistant animals [11]. Different authors suggest the possible upregulation of compensatory mechanisms necessary to regenerate hydroxy-vitamin K in order to maintain the activity of clotting factors.

Recently, the gene encoding the VKOR, or at least an essential protein of its enzymatic activity (vitamin K epoxide reductase subunit 1) (VKORC1), has been
to the lite D1Rat219, it was assumed that it might correspond
this gene is very closed to (about 1 Mb) the microsatel-
cloned and sequenced in humans and rats [12]. Because
ual interest is the observation that the Y139F muta-
tions in warfarin-resistant rats. Of partic-
the reaction solution contained 2 mM dithiothreitol
isolation system (Promega, Charbonnières les Bains, France). DNA concentrations were evaluated spectrophotometrically at 260 nm. The 260/280 ra-
tions were between 1.8 and 2. PCR was performed with 1 μg of extracted genomic DNA using specific primers of rat VKORC1 (VKORC1 GenBank accession # NM-203335). The sequence of the sense primer VKORC1-S1 and the antisense primer VKORC1-AS1 was (5′-GGTTCCTCCCCCTGGGTTGCTG-3′) and (5′-ACTTGGGCAAGGCTCATGTG-3′), respectively. Amplification was performed at 94°C for 90 s, 29 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 3 min, followed by a final extension at 72°C for 5 min. The size of the amplified fragment was 2300 base pairs (bp). Sequencing of PCR products was systematically done (GenomExpress, Meylan, France).

VKORC1 Genotyping

Genomic DNA was extracted from susceptible and resistant rat liver samples by using DNA genomic isolation system (Promega, Charbonnières les Bains, France). DNA concentrations were evaluated spectrophotometrically at 260 nm. The 260/280 ratios were between 1.8 and 2. PCR was performed with 1 μg of extracted genomic DNA using specific primers of rat VKORC1 (VKORC1 GenBank accession # NM-203335). The sequence of the sense primer VKORC1-S1 and the antisense primer VKORC1-AS1 was (5′-GGTTCCTCCCCCTGGGTTGCTG-3′) and (5′-ACTTGGGCAAGGCTCATGTG-3′), respectively. Amplification was performed at 94°C for 90 s, 29 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 3 min, followed by a final extension at 72°C for 5 min. The size of the amplified fragment was 2300 base pairs (bp). Sequencing of PCR products was systematically done (GenomExpress, Meylan, France).

Kinetics Studies on Vitamin K Epoxide Reductase

Vitamin K epoxide (vitamin KO) was prepared from vitamin K (Sigma Aldrich, France) according to [17]. Microsomal vitamin K epoxide reductase (VKOR) activity was assayed as described in [18,19,20], except that the reaction solution contained 2 mM dithiothreitol (DTT), and buffer solution was 200 mM Tris-HCl/0.15 M KC1 buffer (pH 7.4). Thus 0.8 mg of protein was added (method linearity was verified between 0.3 and 1.5 mg of microsomal protein). VKOR activity was assayed at 25°C. The reaction was started by the addition of 20 μM vitamin KO solution in 1% Triton X-100. All incubations were performed in triplicate. The incubation time was 20 min, and the reaction was stopped by addition of 4 mL of iced 1:1 isopropanol/hexane solution. After centrifugation (5000 x g, 5 min), the hexane layer was removed and was dried under nitrogen. The dry residue was immediately dissolved in 0.2 mL of isopropanol and stored at 4°C. Vitamin K was measured by high pressure liquid chromatography (HPLC) (D7000 HS Hitachi AS 2000 automatic injector, injection volume of 50 μL, L 7100 pump, L 4000 UV detector). Separation was achieved on a LiChropher 100 RP18e,
Semiquantitative RT-PCR of VKORC1 mRNA

Total RNA was extracted from susceptible and resistant rat liver samples by means of SV Total RNA isolation system (Promega, Charbonnières les Bains, France). Total RNA concentrations were evaluated spectrophotometrically at 260 nm. The 260/280 ratios were between 1.8 and 2. First strand cDNA templates were synthesized from total RNA (1 µg) in 20 µL of standard reverse transcription buffer (50 mM Tris-HCl pH 8.3, 3 mM MgCl₂, 75 mM KCl, 10 mM dithiothreitol, and 200 µM of each deoxynucleotide triphosphate). After an initial denaturation step at 70 °C for 2 min, 200 units of Moloney Murine Leukemia Virus reverse transcriptase RNAse H minus (MMLV-RT) (Promega, France) were added, and the reaction was incubated at 37 °C for 1 h 30 min, and then at 70 °C for 5 min. Reverse transcriptions of the polyadenylated RNAs were simultaneously performed on all animals of the experiment (3–4 resistant and 3–4 susceptible rats). Apparent $K_m$ and reaction velocity ($V_m$) values for the substrate vitamin KO (12.5–200 µM) were calculated, using microsomes from a pool of 13 susceptible rats or 13 resistant rats. Apparent $K_i$ was determined by incubating various amounts of vitamin KO in the presence of 0, 1, 2, or 5 µM warfarin when microsomes from susceptible rats were used and 0, 20, or 50 µM warfarin when microsomes from resistant rats were used. Data were fitted by a nonlinear regression to the Michaelis–Menten model using the R program developed by Ihaka and Gentleman.

RESULTS

Genotyping the VKORC1 Gene

VKORC1 gene, from 400 bp upstream exon 1 to 100 bp downstream the last exon (exon 3), was sequenced in three warfarin-susceptible and warfarin-resistant rats (Figure 1). The coding sequence was identical in our susceptible rats to the published sequence (NCBI, Genbank NM203335) [12]. In the resistant rats, a unique single nucleotide polymorphism (A415T) was observed and corresponded to the Y139F mutation previously described [13]. This mutation is located in exon 3.

The genotype of all warfarin resistant or susceptible rats used in the catalytic study was verified by direct sequencing of the exon 3. Conditions for the sequencing allowed us to discriminate between homozygous and heterozygous rats (results not shown). Rats homozygous for the mutation or for the wild type were used only.

![FIGURE 1. Partial published VKORC1 sequence (Genbank accession #NM-203335), sequence of genomic DNA encoding VKORC1 in our warfarin-susceptible and resistant rat strains.](image-url)
Kinetics Parameters of VKOR Activity

When VKOR was measured in the presence of 20 μM of vitamin KO, this activity in Sprague-Dawley rats (0.66 nmol/mg protein/min ± 0.022 (mean ± 95% CI), n = 10) was similar to VKOR activity measured in wild susceptible rats (0.62 ± 0.035 nmol/mg protein/min). In resistant rats, VKOR activity was lower (0.22 ± 0.033 nmol/mg protein/min) and statistically different (Student t-test: p < 0.01) when compared with susceptible rats.

Kinetics parameters were analyzed by the measurement of the velocity of the reaction as a function of the KOX concentration. Results obtained for susceptible and resistant rats are presented in Figure 2.

Apparent $V_{\text{max}}$ was 0.67 nmol/min/mg (95% confidence interval: 0.45–0.70 nmol/min/mg) and 0.29 nmol/min/mg (95% CI: 0.27–0.30 nmol/min/mg) in susceptible and resistant rats, respectively. Apparent $K_m$ values were highly different between these two strains (i.e., 57.7 μM (CI 45.3–70.4 μM) and 19.5 (CI 15.4–23.5 μM) in susceptible and resistant rats, respectively).

Inhibition by warfarin of VKOR activity was studied using either microsomes from susceptible rats or from resistant animals. The double reciprocal plots of the velocity of the VKOR activity versus substrate concentration in the presence of different concentrations of warfarin are presented in Figure 3. When microsomes from susceptible rats (Figure 3A) were used, warfarin concentrations were 0, 1, 2, and 5 μM. When microsomes from resistant rats were used, warfarin concentrations were 0, 20, and 50 μM (Figure 3B). Apparent $K_m$

FIGURE 2. Vitamin K epoxide reductase activity versus various amounts of vitamin KO (12.5–200 μM) in susceptible and resistant rats. Microsomes, incubated in triplicates, were from pooled livers from 13 susceptible and 13 resistant rats. Data were fitted by a nonlinear regression, and kinetics constants were calculated for each curve.

was not modified by warfarin in either resistant or susceptible rats. On the contrary apparent $V_{\text{m}}$ was lowered by the use of warfarin. This result confirms that warfarin is a noncompetitive inhibitor of VKOR activity. Data were fitted to the Michaelis–Menten model, which takes into account the presence of either a competitive, a noncompetitive, or an uncompetitive inhibitor by nonlinear regression. A fit was possible when the model that takes into account a noncompetitive inhibitor was used only. $K_i$ were obtained using a nonlinear fitting of the data with S (substrate concentrations) and I (warfarin concentrations) as controlled variables. $K_i$ were 0.72 μM (95% CI 0.67–0.79 μM) and 29 μM (95% CI 24.9–33.1 μM) in susceptible and resistant rats respectively. Values of $V_{\text{m}}/K_m$ were also calculated and are presented in the Table 1.

FIGURE 3. (A) Double reciprocal plots of vitamin K epoxide reductase activity versus vitamin KO (12.5–200 μM), in the presence of 0, 1, 2, and 5 μM warfarin incubated with pooled microsomes from susceptible rats. Inhibition constant ($K_i$) was deduced from each curve. (B) Double reciprocal plots of vitamin K epoxide reductase activity versus vitamin KO (12.5–200 μM) in the presence of 0, 20, and 50 μM warfarin incubated with pooled microsomes from resistant rats. Inhibition constant ($K_i$) was deduced from each curve.
TABLE 1. Kinetic Constants for the Vitamin K Epoxide Reductase from Susceptible and Warfarin-Resistant Rat Strains

<table>
<thead>
<tr>
<th>Rats</th>
<th>(K_m) (µM)</th>
<th>(V_{max}) (nmol/min/mg)</th>
<th>(K_i) (µM warfarin)</th>
<th>(V_{max}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible rat</td>
<td>57.7 ± 12.5</td>
<td>0.67 ± 0.12</td>
<td>0.72 ± 0.06</td>
<td>0.012</td>
</tr>
<tr>
<td>Resistant rat</td>
<td>19.5 ± 4</td>
<td>0.29 ± 0.02</td>
<td>29 ± 4.1</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Values are means ± 2 SD obtained with microsomes pooled from 13 susceptible and 13 resistant rats.

**Expression of VKORC1 mRNA**

The mechanism underlying the low VKOR activity (low \(V_{max}\)) observed in resistant rats in comparison to susceptible rats was investigated by the evaluation of the VKORC1 mRNA expression. Results of the semi-quantitative RT-PCR of the VKORC1 for three warfarin-susceptible and three resistant rats are shown in Figure 4. While GAPDH mRNA expression was similar between warfarin-susceptible and warfarin-resistant rats, results showed a lower expression of VKORC1 mRNA in resistant rat livers as compared with susceptible rats (corresponding signals (mean arbitrary units ± SD) were 72 ± 7 and 151 ± 12, respectively).

**DISCUSSION**

Resistance to antivitamin K compounds, such as warfarin, has been observed in several wild rat strains. On the basis of the catalytic properties described for these resistant rat strains, different mechanisms are possible [13,18].

A common feature observed in different rat strains resistant to AVK is a low level of the in vitro VKOR activity [11,20,21] except in the Scottish resistant rat strain. Indeed, the VKOR activity of Scottish resistant rats is similar to the VKOR activity observed in both susceptible wild rats and Wistar rats [11,22]. Such a weak activity is observed in our resistant rat strain. Due to the involvement of VKOR in the metabolic cycle of vitamin K during which the activation of different clotting factors is achieved, a low VKOR activity should limit the regeneration of vitamin K from the corresponding epoxide. Consequently, a low VKOR activity should reduce the ability of the vitamin K cycle to activate clotting factors. A low VKOR activity could also be linked to increase dietary requirements of vitamin K as observed in different resistant rat strains [23]. In another way, an upregulation of a compensatory pathway, which could be able to regenerate vitamin K hydroquinone from vitamin K epoxide, has been hypothesized.

A first molecular model for warfarin resistance was proposed by Wallin et al. [24] and Wajih et al. [25] in order to describe the resistance of a Chicago rat strain. This model implies an overexpression of calumenin (chaperone protein of the endoplasmic reticulum) observed by western blotting in the liver of resistant rats. This overexpression was shown to be due to an increase of the calumenin mRNA expression as shown by northern blotting. This model explains both the limited VKOR activity and the low inhibitory effect of warfarin on this enzymatic activity.

Earlier genetic studies showed that resistance was frequently linked, in rats, to the \(Rw\) locus, which was located on chromosome 4 and, further, located near the locus D1rat219. Close to this locus, the gene encoding VKOR, or at least an essential protein involved in this enzymatic activity was sequenced. Four mutations are described in human beings resistant to warfarin, and nowadays seven mutations are observed in this gene in resistant rats. The mutation Y139F is shown, when VKORC1 is overexpressed in HEK293, to provoke the most important resistance to warfarin.

Our resistant rat strain presents the Y139F mutation, and most animals are homozygous for this mutation. No other mutation was detected in the sequenced part of the gene (from 100 bp upstream to the ATG to 100 bp downstream to the stop codon). This strain has been maintained in outdoor enclosures for about
15 years. The diet of these animals corresponds to an industrial standard food. The reproduction success is similar to that of the susceptible strain, and no traces of hemorrhages have ever been observed during necropsy. No clinical signs of vitamin K deficiency have ever been observed either.

The VKOR activity from homozygous resistant rats is low as observed in other strains (\( V_{\text{max}} \) is 0.29 ± 0.02 nmol/min/mg). The low \( K_m \) observed in resistant rats, in comparison to susceptible animals, results of an enzymatic efficiency \( V_{\text{max}}/K_m \) very similar or even higher to the value obtained with susceptible rats (Table 1). This identical enzymatic efficiency is probably a good explanation for the absence of clinical signs of vitamin K deficiency. Indeed, for physiological concentrations of vitamin KO, the regeneration of vitamin K quinone remains strictly efficient. This compensatory mechanism is an unexpected consequence of the mutation. Indeed, only an absence of inhibition by warfarin was reported for this mutation so far. As a consequence, we hypothesize that the decrease in the apparent \( V_{\text{max}} \) might be an adaptation to the low apparent \( K_m \) in order to maintain the enzymatic efficiency \( (V_{\text{max}}/K_m) \) in the usual values. The corresponding decrease in the concentration of the mRNA encoding for VKORC1 argues for this hypothesis. Such a decrease might be due to RNA instability and, in this case, such a correlated decrease might be merely, although unlikely, coincidental, or this RNA decrease might be explained by a translational regulation of the VKOR expression. This last hypothesis is currently studied in our laboratory.

The inhibiting effect of warfarin is very low in our resistant rat strain compared to susceptible rats or laboratory rats. The \( K_i \) observed is 42-fold greater in resistant animals than in control rats. This point is in good agreement with in vitro consequences of the Y139F mutation. Such a high \( K_i \) provides a good explanation to the in vivo resistance observed in our rat strain. The involvement of Y139 in catalytic properties of the VKORC1 seems to be of great importance [26]. Nevertheless if one considers that the interaction site for a noncompetitive inhibitor is different from the site of interaction for the substrate, it is difficult to postulate that this tyrosine is involved in these two sites. It remains probable that this tyrosine is necessary for the interaction with the inhibitor but that the mutation modifies indirectly the conformation of the substrate site increasing the affinity for the substrate.

Furthermore, this complex genetic basis should take into account the behavioral aspects (and their potential genetic bases) that are of great importance in the global expression of the resistance in a population of wild rats.

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REFERENCES