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WARFARIN RESISTANCE IN A CHICAGO STRAIN OF RATS

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Abstract—A warfarin-resistant strain of rats trapped in Chicago was studied to determine the mechanism of the warfarin resistance. The Chicago-resistant rats (CR) differ from a Welsh-resistant strain (WR) which has a vitamin K epoxide reductase that is insensitive to warfarin. The epoxide and dithiol-dependent quinone reductases of the CR rats were as sensitive to warfarin as the normal enzyme. Unlike the irreversible warfarin inhibition seen in normal rats, the warfarin inhibition of the epoxide reductase from the CR strain was partially reversible *in vitro*. In this respect, the CR rats appeared similar to a Scottish warfarin-resistant strain. The same steady-state level of warfarin (40 ng/mg protein) in liver microsomes could be achieved in normal and CR strain rats following a few days ingestion of a diet containing 50 ppm warfarin, but clearance of warfarin (1 mg/kg) from the liver microsomes was more rapid in the CR strain than in normal rats, and the recovery of epoxide reductase activity and prothrombin levels was more rapid. The mechanism of warfarin resistance in the CR strain differed from the warfarin resistance mechanisms of both the Scottish- and Welsh-resistant rat strains. The combination of an increased rate of warfarin clearance and the partially reversible inhibition of the epoxide reductase would be sufficient to allow the rats to survive a limited exposure to warfarin.

Substituted 4-hydroxycoumarins such as warfarin (3-α-acetonylbenzyl-4-hydroxycoumarin) are vitamin K antagonists and are used extensively as both clinical anticoagulants and rodenticides. The reduced form of vitamin K is a substrate for an enzyme catalyzing the posttranslational carboxylation of specific glutamyl residues in intracellular precursors of a limited number of proteins to γ-carboxyglutamyl residues in biologically active proteins [1]. These proteins include the plasma coagulation factors II (prothrombin), VII, IX, and X, and warfarin administration causes a decrease in the activities of these proteins and a hemorrhagic condition. The action of the carboxylase also produces vitamin K-2,3-epoxide as a product. Warfarin inhibits a dithiol-dependent vitamin K epoxide reductase and a dithiol-dependent vitamin K quinone reductase which function to reduce the epoxide and to regenerate the active cofactor form of the vitamin [2–4].

The extensive use of warfarin as a rodenticide has led to the selection of warfarin-resistant rat strains in a number of geographic locations [5]. The most extensively studied of these is a Welsh strain which has been shown to have an epoxide reductase and a quinone reductase that are much less sensitive to warfarin than the enzymes from normal rats [3]. More recently, warfarin resistance has been shown to be widespread in the Chicago area of Illinois, and this report describes the results of studies of the mechanism of resistance in this strain of rats.

MATERIALS AND METHODS

Animals. Male or female 250 g rats, obtained from Sprague–Dawley (Madison, WI), were used as normal controls. Chicago warfarin-resistant strain male or female rats (CR) were obtained from BioCenotics (Osseo, MI). Wild warfarin-resistant rats were trapped in Chicago and crossed with golden rats to produce a golden-resistant strain. Both wild and golden CR rats were used in this study. The type of rat used in each experiment is indicated in the figure legend, and the wild CR rats were used unless golden is indicated. The Welsh strain warfarin-resistant rats (WR) were raised in a breeding colony maintained at the University of Wisconsin-Madison. They were fed commercial Purina rat chow and supplemented with 1 mg/L menadione sodium bisulfite in the drinking water.

Tissue preparation. Control and experimental animals were anesthetized with ether and killed by decapitation, and livers were excised, rinsed, and weighed. Liver microsomes were prepared by a 50% (w/v) homogenate of the livers in 0.25 M sucrose/0.025 M imidazole (pH 7.2) buffer (SI buffer) using a Potter–Elvehjem homogenizer (teflon pestle) to rupture cells. The homogenates were centrifuged at 10,000 *g* for 15 min using a Beckman JA-21 rotor, and the postmitochondrial supernatant was centrifuged at 105,000 *g* for 60 min using a Ti65 ultracentrifuge rotor. The microsomal pellets were surface washed with SI buffer and stored at −70°. Blood samples (1 mL) for prothrombin assay were obtained by cardiac puncture of etherized animals using a syringe containing 0.1 mL of 2.85% sodium citrate to prevent coagulation. Plasma was obtained after centrifuging blood samples for 15–20 min at 1500 rpm, 4°.

Assays. Unless otherwise noted, vitamin K epoxide reductase activity was assayed at 25° in 0.01 M *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS)/0.25 M sucrose/0.15 M KCl, pH 8.8 (TAPS buffer) containing 1 mM dithiothreitol (DTT), 40 μM vitamin K epoxide in 1% Emulgen

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911, and 0.25 mL liver microsomes solubilized in TAPS buffer containing 0.3% sodium cholate. The 0.5-mL assay mixtures were quenched after 5 min with 1.0 mL isopropanol/hexane (3/2, v/v). The hexane layer was removed, dried under nitrogen, and dissolved in 0.2 mL methanol. Vitamin K was quantitated by HPLC using a Waters (Milford, MA) model 712 WISP automatic injector, M-45 pump, 440 absorbance detector, and 730 data processor. Separation was achieved on a Waters C18 μBondapak 10 μm analytical column run at 2 mL/min in 95% methanol/5% water. External standard quantitation was based on integrated absorbance at 254 nm. Extinction coefficients of 6170 M^{-1} cm^{-1} at 259 nm for vitamin K epoxide and 18,900 M^{-1} cm^{-1} at 248 nm for vitamin K were used [6]. Substrate K_m values were calculated by fitting initial rate data to the hyperbola $v = VA/(K + A)$ using Cleland's program HYPERO [7].

Dithiol-dependent vitamin K quinone reductase activity was assayed at 25° in TAPS buffer containing 1 mM DTT, 40 μM vitamin K (in 1% Emulgen 911) and 0.25 mL microsomes solubilized in 0.3% cholate/TAPS buffer. The 0.5-mL assay was conducted in vials that were purged under nitrogen in order to prevent oxidation of the reduced vitamin K (vitamin KH_2) that is produced. After 5 min at 25° the assay was quenched with 2 mL isopropanol, and the protein was pelleted by a brief centrifugation. A 500-μL aliquot of the aqueous layer was analyzed immediately for vitamin KH_2 content on an HPLC system using fluorometric detection (excitation at 340 nm and emission at 430 nm). Quinone reductase activity was expressed as peak heights of vitamin KH_2 formed (1 unit = 1 cm).

The reversibility of warfarin inhibition of epoxide reductase activity was assayed in a manner similar to that described by Thijssen [8]. Microsomes resuspended in 0.02 M Tris/0.15 M KCl/0.25 M sucrose (pH 7.4) (Tris buffer) were incubated with 1 μM sodium warfarin for 10 min at room temperature, diluted 10-fold with Tris buffer ± 2 mM DTT, and incubated for another 10 min at room temperature. Centrifugation at 105,000 *g* for 60 min at 4° in a Sorval model OTD75B ultracentrifuge utilizing a Ti65 rotor pelleted the microsomes. Epoxide reductase activity of the pellet resuspended in Tris buffer was assayed for 10 m in at 25° in the presence of 1 mM DTT and 20 μM vitamin K epoxide in 1% Emulgen 911.

Microsomal warfarin content was determined by HPLC analysis using DL-3-(acetonyl-4-chlorobenzyl)-4-hydroxycoumarin (*p*-Cl-warfarin) as an internal standard. Microsomal pellets were resuspended in SI buffer and mixed with an equal volume of acetonitrile and 2 vol. of citric acid/phosphate buffer (pH 4.2) prior to two extractions with methylene chloride/pentane (1/1, v/v) followed by an extraction of the combined organic layer with 0.2 mL of 0.5 M HCl [9, 10]. The organic layer was dried under nitrogen or air and dissolved in 0.2 mL eluent [1.5% acetic acid, pH 4.7/acetonitrile (65/35, v/v)]. Separation was achieved on a Waters C18 μBondapak 10 μm analytical column run at 2 mL/min in the eluent. External standard quantitation was based on integrated absorbance at 313 nm.

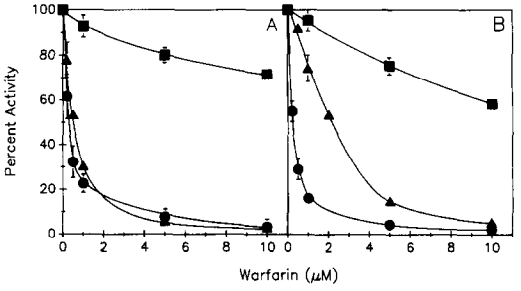

Fig. 1. Effects of warfarin on vitamin K epoxide reductase (A) and dithiol-dependent vitamin K quinone reductase (B) activities in hepatic microsomes from normal (—▲—), Chicago-resistant (CR) (—●—), and Welsh-resistant (WR) (—■—) rats. Assay conditions are described in Materials and Methods. Each point and vertical bar represent the mean ± range of two experiments, expressed as a percentage of the activity in the absence of warfarin. The control epoxide reductase activity equalled 0.76 ± 0.02, 0.51 ± 0.02, and 0.500 ± 0.002 nmol vitamin K/min for the normal, CR, and WR microsomes respectively. The control quinone reductase activity equalled 4.1 ± 0.2, 3.1 ± 0.2, and 3.5 ± 0.2 units/min for the normal, CR, and WR microsomes.

Protein was determined using the Bradford assay [11] with bovine serum albumin as the standard.

Plasma prothrombin was determined by assay of thrombin-catalyzed amidolysis of a chromogenic peptide substrate following thromboplastin activation [12]. Vitamin K-dependent carboxylase activity was assayed as described previously [13].

Chemicals. Vitamin K (phylloquinone) was converted to vitamin K epoxide by the method of Tischler *et al.* [14], and the epoxide was purified by preparative HPLC using a Waters C18 μBondapak 10 μM semipreparative column run at 4 mL/min in 100% methanol. Vitamin K and TAPS were purchased from Sigma (St. Louis, MO). Emulgen 911 was from KAO Atlas (Tokyo, Japan), DTT and Tris were from Boehringer Mannheim (Indianapolis, IN), sodium cholate was from Kodak (Rochester, NY), *p*-Cl-warfarin was from Aldrich (Milwaukee, WI), and sodium warfarin was obtained from the Wisconsin Alumni Research Foundation (Madison, WI). HPLC grade solvents were from American Burdick & Jackson (Muskegon, MI).

RESULTS

The different nature of the resistance to warfarin in the Welsh-resistant (WR), Chicago-resistant (CR), and normal Sprague–Dawley strains of rats is evident in Fig. 1. Assays of vitamin K epoxide reductase activity in the livers of these three strains of rats indicated that the sensitivity of this enzyme to warfarin was identical in the normal and CR strains but greatly reduced in the WR strain. A similar experiment utilizing liver microsomes from the golden CR strain (data not shown) yielded identical results. The sensitivity of the vitamin K quinone reductase in the WR rats was also greatly decreased from that seen in the normal or CR rats. The WR strain has been shown previously [15–17]

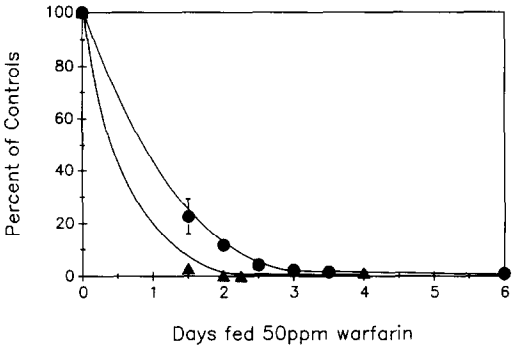

Fig. 2. Effect of warfarin ingestion on plasma prothrombin concentration. Normal (—▲—) and Chicago-resistant (—●—) female rats were fed a diet containing 50 ppm warfarin for up to 6 days (the same rats were used in Figs. 2–4). Each point and vertical bar represent the mean ± SE of three rats from one representative experiment. Prothrombin concentration is expressed as a percentage of the concentration in control rats of each strain fed commercial rat chow. The plasma prothrombin concentration of CR rats was 113 ± 6% that of a pool of normal rat plasma.

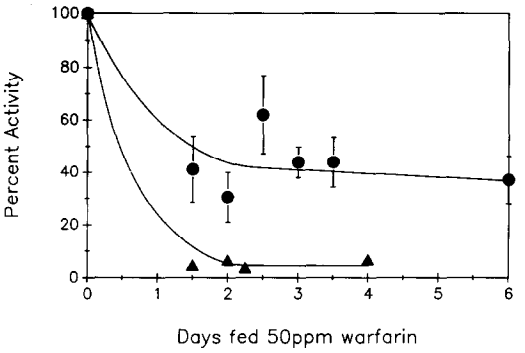

Fig. 3. Effect of warfarin ingestion on vitamin K epoxide reductase activity. Normal (—▲—) and Chicago-resistant (—●—) female rats were fed a diet containing 50 ppm warfarin for up to 6 days (the same rats were used in Figs. 2–4). Each point and vertical bar represent the mean ± SE of three rats from one representative experiment. The epoxide reductase activity is expressed as a percentage of the activity in control rats fed commercial rat chow (0.26 ± 0.01 and 0.042 ± 0.001 nmol vitamin K/min for normal and Chicago-resistant rats respectively).

to have an increased requirement for vitamin K and must be maintained on a high vitamin K diet. In contrast, the CR strain rats were found to maintain normal plasma prothrombin concentrations when fed commercial rat chow.

A second possible mutation that would result in a phenotypic resistance to warfarin would be an alteration of the vitamin K-dependent carboxylase to a form of the enzyme with a much lower K_m for reduced vitamin K (vitamin KH_2). Normal rates of carboxylation could then proceed in the presence of the lower amount of vitamin KH_2 which would be present in microsomes if the epoxide and quinone reductases were inhibited by warfarin. The apparent K_m for vitamin KH_2 of the microsomal vitamin K-dependent carboxylase from the normal strain of rats was found to be 27 ± 3 μM, while the enzyme from the CR strain had a K_m of 29 ± 7 μM.

The standard World Health Organization protocol to determine warfarin resistance is to feed rats a no choice 50 ppm warfarin diet for 6 days [18]. Rats which survive are considered resistant. When normal rats were subjected to this protocol, plasma prothrombin concentration decreased to less than 5% of starting concentration at 36 hr, and all rats died by day 6 (Fig. 2). All of the CR rats subjected to this protocol survived, even though plasma prothrombin concentrations following day 3 were less than 5% of normal. When the liver microsomes of these rats were assayed for epoxide reductase activity, it was found to decrease rapidly to less than 5% of the initial activity in the normal rats, but to remain at about 40% of initial values in the CR rats (Fig. 3). The data in Fig. 4 indicate that although epoxide reductase activities differed markedly, essentially the same hepatic concentration of warfarin was reached in both strains of rats when they were fed 50 ppm warfarin in their diet.

It is unlikely that a rodenticide-baited wild rat population is subjected to a continued uniform

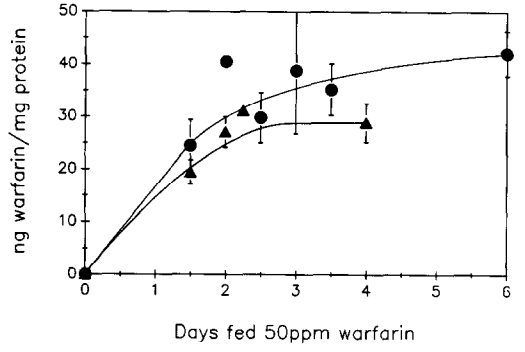

Fig. 4. Effect of warfarin ingestion on liver microsomal warfarin content. Normal (—▲—) and Chicago-resistant (—●—) female rats were fed a diet containing 50 ppm warfarin for up to 6 days (the same rats were used in Fig. 2–4). Each point and vertical bar represent the mean ± SE of three rats from one representative experiment.

exposure to warfarin, and it is more likely that large, often toxic, doses are ingested, followed by periods where no warfarin is consumed. The data in Figs. 5–7 illustrate the response of normal, WR, and CR strain rats to a single 1 mg/kg, i.p., injection of warfarin. Prothrombin concentrations and epoxide reductase activity in WR were unaffected by this treatment (Figs. 5 and 6). In normal rats, plasma prothrombin concentrations decreased to 10% of initial values by 2 days and then began to recover (Fig. 5). In CR rats, prothrombin concentration showed a decline to a minimum of 40% at 1 day and had returned to normal levels by day 4. Epoxide reductase activities (Fig. 6) showed a similar response. The activity of the enzyme in WR rats was unaffected, the CR rats showed an initial decline in epoxide reductase activity and a rapid return to normal, and the normal rats demonstrated a rapid

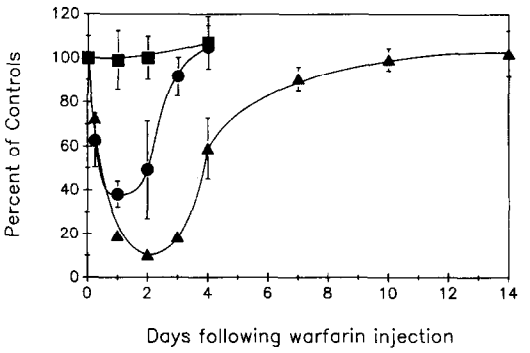

Fig. 5. Effect of warfarin injection on plasma prothrombin concentration. Normal (—▲—), golden Chicago-resistant (CR) (—●—), and Welsh-resistant (WR) (—■—) female rats were injected with 1 mg/kg sodium warfarin (i.p.) and killed at various times after the warfarin injection (the same rats were used in Figs. 5–7). Each point and vertical bar represent the mean ± SE of three rats from one representative experiment. The plasma prothrombin concentration of CR rats was 104 ± 15% that of a pool of normal rat plasma, while the WR rat plasma was 114 ± 13% of the normal plasma.

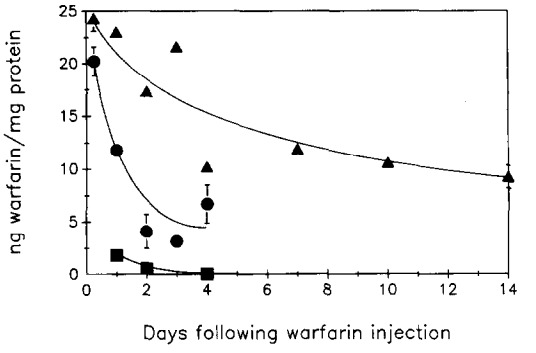

Fig. 7. Effect of warfarin injection on liver microsomal warfarin content. Normal (—▲—), Welsh-resistant (WR) (—■—), and golden Chicago-resistant (CR) (—●—) female rats were injected with 1 mg/kg sodium warfarin (i.p.) and killed at various times after the warfarin injection; then microsomal warfarin content was measured (the same rats were used in Figs. 5–7). Each point and vertical bar represent the mean ± SE of three rats from one representative experiment.

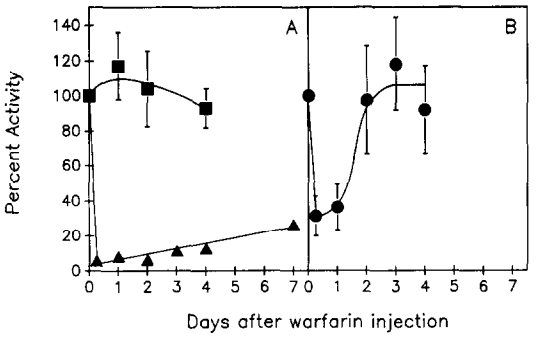

Fig. 6. Effect of warfarin injection on vitamin K epoxide reductase activity. Normal (—▲—), Welsh-resistant (WR) (—■—) (panel A), or golden Chicago-resistant (CR) (—●—) female rats (panel B) were injected with 1 mg/kg sodium warfarin (i.p.) and killed at various times after the warfarin injection (the same rats were used in Figs. 5–7). Each point and vertical bar represent the mean ± SE of three rats from one representative experiment. Vitamin K epoxide reductase activity in hepatic microsomes is expressed as a percentage of the activity in saline-injected controls from each strain (0.32 ± 0.03, 0.11 ± 0.04, and 0.18 ± 0.02 nmol vitamin K/min for the normal, CR, and WR microsomes respectively).

loss of activity of this enzyme, and a very slow recovery. The data in Fig. 7 illustrate that warfarin did not accumulate to a significant extent in WR rat liver, had a half-life of about 1 week in normal rats, and disappeared very rapidly from the liver of CR rats after a transient rise.

The possibility that the mutation responsible for warfarin resistance in the CR rats had altered the epoxide reductase in ways not related to its warfarin sensitivity was also considered. Kinetic constants for the enzyme activity measured in normal and CR strain rats are shown in Table 1. The K_m values for vitamin K epoxide were similar in the two strains of rats, but the K_m for the second substrate, DTT, was increased in the CR strains. The V_{max} for epoxide reduction was also halved in the CR strain.

A resistant strain of rats located in Scotland has been shown to have epoxide and quinone reductases as sensitive to warfarin inhibition as the normal strain. However, the inhibition of both reductases by warfarin is readily reversible [8, 19]. Washing warfarin-treated microsomes from this strain with buffer plus 2 mM DTT restores 100% of the epoxide reductase activity, while 76% of the activity is restored after washing with buffer alone [8]. The data in Table 2 indicate that the enzyme from the CR strain recovered only 67% of its activity after washing with 2 mM DTT, and 54% of the activity upon washing with buffer alone. Equal concentrations of microsomal warfarin were also seen after washing with or without 2 mM DTT. Less activity was recovered when normal microsomes were washed, consistent with the higher concentration of warfarin that was retained in the microsomes. Similar results (data not shown) were obtained when liver microsomes from golden CR rats were utilized.

DISCUSSION

The biochemical basis for warfarin resistance in a strain of rats trapped in Chicago, Illinois, has been studied and compared to the resistance of a widely studied Welsh-resistant strain. In contrast to the drastically decreased warfarin sensitivity of the vitamin K epoxide reductase and vitamin K quinone reductase of the WR strain, the *in vitro* sensitivity to warfarin of these enzymes was not altered in the CR strain. The apparent K_m for vitamin K epoxide of the CR strain was similar to that of the normal epoxide reductase, whereas the apparent K_m for DTT was elevated by 2.7 times. The CR enzyme was less active, however, with a V_{max} of 0.12 nmol

Table 1. Kinetic constants for epoxide reductase from normal and CR strain rats

Rats	K_m KO	K_m DTT	V_{max} (nmol vitamin K/min)
Normal	21 ± 5 μM	0.10 ± 0.03 mM	0.33 ± 0.02
Chicago-resistant	12 ± 3 μM	0.27 ± 0.03 mM	0.12 ± 0.01

Apparent K_m for vitamin K epoxide (KO) was measured in the presence of 1 mM dithiothreitol (DTT) and apparent K_m for DTT was measured in the presence of 40 μM KO. Values are means ± SE for three experiments (V_{max} and K_m for KO) or mean ± range for duplicate experiments (K_m for DTT).

Table 2. Effect of washing on vitamin K epoxide reductase activity and warfarin content in microsomes of normal or Chicago-resistant (CR) rats

Wash procedure	Percent of uninhibited control: Normal	Percent of uninhibited control: CR	Warfarin (ng/mg protein): Normal	Warfarin (ng/mg protein): CR
None	18 ± 8	27 ± 1	13.7 ± 0.7	13.8 ± 0.8
Tris buffer	23 ± 4	54 ± 5	5.6 ± 0.2	3.6 ± 0.1
Tris buffer + 2 mM DTT	26 ± 6	67 ± 4	6.1 ± 0.4	4.0 ± 0.1

Microsomes pooled from twelve rats of each strain were incubated in 1 μM warfarin for 10 min and assayed directly or diluted 10-fold and washed by repelleting and resuspension as described in Materials and Methods. Activity is expressed relative to uninhibited microsomes and has been corrected for the 35–40% loss of activity that was observed in uninhibited controls upon washing and resuspension. Values are means ± SE for six assays of epoxide reductase activity, and duplicate assays ± range for warfarin concentration.

vitamin K/min as opposed to 0.33 nmol K/min for the normal enzyme. More importantly, *in vitro* warfarin inhibition of the CR epoxide reductase appeared to be partially reversible rather than irreversible as is the case with the normal enzyme. In this respect, the CR rat strain is similar to a third, Scottish-resistant, strain in which inhibition of the epoxide reductase is reversed by washing warfarin-treated microsomes with 2 mM DTT. The CR strain, however, did not recover all of its epoxide reductase activity after washing, and the presence of DTT did not make a difference in the amount of activity recovered. Therefore, the Scottish and Chicago strains appear to have similar but somewhat different resistance mechanisms.

A major factor in the warfarin resistance of the Chicago strain appeared to be the rapid disappearance of warfarin from the liver. After a 1 mg/kg injection of warfarin, half of the warfarin was cleared from the livers of the Chicago strain within 1.5 days as opposed to 7 days in normal rats. The same steady-state level of warfarin (40 ng/mg protein) in liver microsomes can be achieved in normal and CR strain rats following a few days ingestion of a diet containing 50 ppm warfarin. The higher activity of the epoxide reductase in the livers of the CR strain compared to normal rats under these conditions (Fig. 3) was due presumably to the partial reversibility of the warfarin inhibition in the CR strain. The WR strain was relatively unaffected by the warfarin diet and did not retain warfarin in the liver microsomes.

The mechanism of warfarin resistance in the Chicago-resistant rat strain is, therefore, complex and appears to differ from the warfarin resistance mechanisms of both the Scottish- and Welsh-resistant rat strains. The vitamin K-dependent carboxylase did not appear to be involved, and both the vitamin K epoxide reductase and dithiol-dependent vitamin K quinone reductase were sensitive to warfarin inhibition. The warfarin inhibition of the epoxide reductase appeared to be partially reversible. While warfarin was retained in the liver of CR rats, the rate of warfarin disappearance from the CR liver microsomes *in vivo* was very rapid compared to that seen in microsomes of normal rats. The increased clearance rate of warfarin alone was not enough to make the rats resistant, since after a few days on the 50 ppm warfarin diet, the steady-state level of warfarin in the livers of the CR rats was the same as in the normal rats. The CR rats may not survive a prolonged exposure to warfarin, but the combination of an increased rate of warfarin clearance and the partially reversible inhibition of the epoxide reductase would be sufficient to allow the rats to survive a limited exposure to warfarin and is the most likely explanation for their survival in a local population subjected to baiting by warfarin.

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