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Warfarin resistance: biochemical evaluation of a warfarin-resistant wild brown rat

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The 4-hydroxycoumarin warfarin has been introduced as a rodenticide in the late 1940s. Its ideal rodenticide properties has made its employment world-wide. An inheritable form of warfarin resistance in wild rats was discovered soon, first in Scotland, later in other countries [1–4].

Growing insight into the biochemical function of vitamin K* and in the interaction of 4-hydroxycoumarins thereupon has been obtained for the last 15 years and the experimental use of homozygous warfarin-resistant laboratory rats contributed a lot to our understanding [5, 6]. These rats were obtained by cross-breeding warfarin-resistant wild rats (*Rattus norvegicus*) trapped in Wales with albino Wistar rats. The biochemical mechanism of that warfarin resistance turned out to be reduced sensitivity of the target enzyme vitamin KO reductase for warfarin [5–7]. The enzyme is part of the so-called cellular vitamin K cycle and regenerates vitamin K from vitamin KO, a metabolic product of the carboxylation reaction that transforms glutamyl residues of substrate proteins into gamma-carboxyglutamyl structures [8, 9]. It is believed that vitamin KO reductase becomes inactivated (= oxidized) during substrate conversion and that a dithiol is involved in its reactivation. This reactivation step is hampered by the bound 4-hydroxycoumarin [10, 11].

Recently, it became apparent that the warfarin resistance of the Welsh genotype is not the only mechanism. MacNicoll [12] showed the *in-vitro* warfarin susceptibility of vitamin KO reductase of Scottish resistant rats does not differ from sensitive rats, and our laboratory showed the mechanism of resistance of the Scottish genotype to be due to a vitamin KO reductase, the dithiol-dependent reactivation of which is not sensitive to warfarin [13].

During 1985 warfarin resistance among wild brown rats in a rural area in the eastern part of The Netherlands was reported. The rats were trapped by local authorities and tested for their resistance according to WHO test directives by the department of "Plague Control" of the Ministry of "Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer".

We investigated the *in vitro* characteristics of the hepatic vitamin KO reductase of these rats to elucidate the type of resistance. The results, reported in the present paper, suggest biochemically their warfarin resistance to be of the Welsh type.

Materials and methods

Rat livers of male Wistar rats and of male warfarin-resistant rats of the Scottish genotype (HS strain [14]) were excised from the rats after exsanguination via the abdominal aorta. Rat livers of male warfarin-resistant rats of the Welsh genotype (HW strain [14]) were a gift of Dr A. D. MacNicoll, Ministry of Agriculture, Fishery and Food, Tolworth Laboratory, U.K. Rat livers of male warfarin-resistant wild brown rats were obtained through the courtesy of Mr DeJonge of the department of "Plague Control", Wageningen, The Netherlands. The livers were from four male rats which survived a 6-day no-choice feeding schedule of 0.025% warfarin containing oats. The tests were performed by the department of "Plague Control". The rats were of a group of animals (seven males, 288 ± 45 g; ten females, 196 ± 51 g) that were trapped in the area known for warfarin resistance. The average daily oats intake during the feeding test was about 13 and 8 g for the males and females, respectively, indicating about 11 and 9.5 mg/kg of warfarin per day. One female animal died of bleeding on day 6 (Mr DeJonge, personal communication). The other ones survived and were observed for another 2 weeks whereafter they were killed. Our laboratory obtained the livers of four male rats.

Liver homogenates were prepared in three times their volume (w/v) 0.02 M Tris buffer, pH 7.4, containing 0.15 M KCl and 0.25 M sucrose by the Potter technique. All manipulations were performed in the cold. The homogenates were pooled, N = 3 for the laboratory strains, N = 4 for the wild rats. Liver microsomes were prepared as described previously [13]. Microsomal vitamin KO reductase activity was assayed as described previously [13], except that vitamin KO (5 mM) in 2% Triton-X 100 in Tris buffer was used as substrate solution instead of an isopropanol stock solution. Thus, 20 μl of microsomes (about 0.3 mg of protein assayed by the Lowry method) and 75 μl Tris buffer were mixed in a reaction vessel. Following a 3-min incubation (30°) period, 2 μl of DTT (0.1 M in Tris buffer) were added and the mixture was incubated for another 3 min. The reaction was started by the addition of 2 μl of the vitamin KO stock solution. All incubations were performed in duplicate. The reaction was stopped by the addition of 0.9 ml of isopropanol. Extraction and analysis by HPLC were as described previously [13]. Further details are given in the text.

Results

Vitamin KO reductase activity of the microsomal preparation of the wild warfarin-resistant rat was comparable to the activity found for the susceptible Wistar strain, respectively 250 ± 50 and 350 ± 55 (mean ± SD, two separate duplicate assays) pmole vitamin K formed per mg protein per min. The activity of the HS strain was 320 pmol (duplicate assay). The activity of the Welsh warfarin-resistant HW strain at the test conditions was much lower: 80 ± 11 (N = 4). The HW microsomes as well as those of the wild rats showed the formation of 3(2)-hydroxy-2,3-dihydrovitamin K (Fig. 1). This metabolite is presumed to be an intermediate in the reaction process [15]. The activity in the wild rats, however, was lower than the HW activity. The *in-vitro* sensitivity of the different vitamin KO reductases for S-warfarin inhibition is depicted in Fig. 2A. At first sight, the data suggested the wild reductase to be sensitive, $I_{50} < 0.4$ μM, albeit that even at elevated (10 μM) S-warfarin concentrations activity was still observable; 35 pmol vitamin K (about 15% of control). For comparison HW activity was 60% (65 pmol vitamin K) and HS activity less than 3% (10 pmol vitamin K) of control activity. At 1.3 μM S-warfarin the activities were 93, 23, 17 and 3% of control, comparable to about 75, 60, 55 and 10 pmole *in vitro* vitamin KO reductase activity, for HW, wild, HS and Wistar, respectively. The fractional resistance of the wild rat was investigated further. Microsomes were incubated with 33 μM S-warfarin (30 min) to titrate the sensitive

* Abbreviations used: vitamin K, vitamin K1; vitamin KO, vitamin K1 2,3-epoxide; DTT, dithiothreitol.

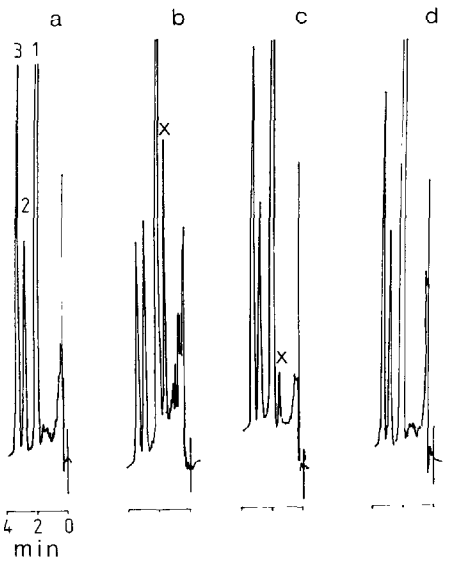

Fig. 1. HPLC analyses of microsomal vitamin KO reductase activity. (a) susceptible Wistar rats; (b) resistant HW rat; (c) resistant wild rat; (d) resistant HS rat. Peaks 1–3 are vitamin KO, internal standard and vitamin K, respectively. Peak X is assigned to 3(2)-hydroxy-2,3-dihydrovitamin K. Incubations contained about 0.3 mg microsomal protein, 2 mM DTT and 100 μM vitamin KO. Reaction time was 5 min.

enzyme. Following a 20-fold dilution to wash out the warfarin the microsomes were recovered by centrifugation. Whereas sensitive microsomes were more than 95% inactivated by this, the wild microsomes retained about 25% of the activity. This residual reductase activity was less sensitive to S-warfarin. An inhibition plateau was observed (Fig. 2B) showing an activity of about 30 pmole vitamin K per mg protein per min.

The Scottish type of resistance was tested for by studying the influence of DTT on the reductase activity in the presence of 1 μM S-warfarin. The rate of vitamin K formation at 2 mM DTT was 28, 64 and 67 pmole per mg protein per min (mean of duplicate experiments) for Wistar, HS, and wild microsomes. Enhancement of DTT to 15 mM increased the reductase activity of the HS microsomes to 240 and of the Wistar microsomes to 86 pmole vitamin K. The activity of the wild microsomes was not affected, excluding the Scottish genotype of resistance. This conclusion was confirmed by wash experiments. Whereas the activity of S-warfarin (10 μM) treated HS microsomes was restored almost completely by washing them in buffer containing 2 mM DTT [13], the reductase activity of the wild rat—which was reduced to about 20% by the warfarin treatment—was not reactivated.

The data so far suggested the mechanism of warfarin resistance of the wild rat either to be completely different from the known mechanisms; for instance, an altered vitamin KO reductase which although fully saturated with warfarin still catalyses the vitamin KO reduction be it at a rate of 15–20%, or, alternatively, we are dealing with at least two (iso)enzymes, a sensitive and an insensitive one. The insensitive enzyme shows characteristics of the Welsh-type vitamin KO reductase; among others, the formation or release of the hydroxyvitamin K metabolite (Fig. 1). This latter explanation is the more attractive one. It also suggests that the wild rat livers were heterozygote with respect to the enzyme.

To test the latter hypothesis, we got the opportunity to investigate the microsomal vitamin KO reductase activity of F_1 litters (three females, two males, about 8 weeks old) born in captivity from a mating of a wild couple (the livers were provided through the courtesy of Mr DeJonge). The data, summarized in Table 1, showed for four of the five siblings a strongly reduced sensitivity for 3.3 μM S-warfarin; 20–30% inhibition. One rat ($F_1$4) was inhibited by about 70%, whereas the susceptible Wistar rat was almost completely inhibited. The formation of the hydroxyvitamin K metabolite was observed for all the F_1s. Inhibition curves showed the HW type of sensitivity (see Fig. 2A) for the F_1s 1–3 and 5; 65–80% control activity at 5 μM S-warfarin. Rat $F_1$4 was comparable to the picture shown for the wild rat in Fig. 2A; 23% control activity at 5 μM S-warfarin. These results strongly suggest the F_1s to be the outcome of a mating between parents homo- and heterozygote for the Welsh-type warfarin-resistant vitamin KO reductase.

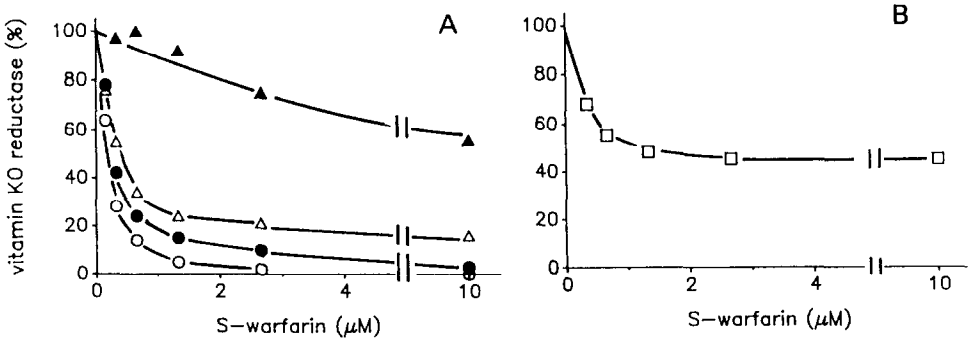

Fig. 2. (A) The effect of S-warfarin on microsomal vitamin KO reductase of Wistar (○), HS (●), wild (△), and HW (▲) rats. The reaction mixtures were pre-incubated in the presence of S-warfarin for 5 min before DTT was added (see Materials and Methods). (B) The effect of S-warfarin on the residual microsomal vitamin KO reductase of S-warfarin titrated wild microsomes (for details, see text).

Table 1. Vitamin KO reductase activity in liver microsomes of F_1 litters of wild warfarin-resistant brown rats*

Rat	Sex	pmol Vitamin K/mg protein/min	
		Control	+ Warfarin†
$F_1$1	M	110	75 (68)
$F_1$2	M	44	34 (78)
$F_1$3	F	125	88 (71)
$F_1$4	F	173	54 (31)
$F_1$5	F	115	80 (70)
Wistar	M	240	9 (4)

* The data are the results of duplicate assays.

† Reductase activity in the presence of 3.3 μM S-warfarin. The numbers in parentheses are % of control values.

Discussion

Warfarin resistance among rodents is a well known phenomenon which imposes great financial as well as health problems for the (agrarian) community. Knowledge of the mechanism of the resistance may add to the successful control of the plague. In this study, we investigated the biochemistry of the vitamin KO reductase of a warfarin-resistant wild brown rat living in a rural area of the eastern part of The Netherlands. Our first experiments were performed with pooled microsomes. The results, when compared to the characteristics of the well documented Welsh [6, 7] or Scottish [11, 13] genotype of warfarin resistance, although not as unambiguous to allow clear-cut interpretations, led us to suggest the vitamin KO reductase of the warfarin-resistant wild rat to be of the Welsh-type and the rats to be heterozygote with respect to that gene. At least two (iso)enzymes vitamin reductase could be traced, a warfarin-sensitive and -insensitive one. The results obtained with the individual livers of five F_1 litters affirmed that conclusion. One of the litters showed the patterns observed for the pooled livers (Fig. 2A). The vitamin KO reductase of the others showed properties comparable to the homozygous HW rat. Typically, all the livers of the wild rats showed the formation of 3(2)-hydroxy-2,3-dihydrovitamin K, a phenomenon characteristic for the Welsh-type of resistant vitamin KO reductase [15]. Another remarkable point of the Welsh-type warfarin-resistant rats is their relatively low vitamin KO reductase activity in comparison to a susceptible rat; 25–50%, this study and that of others [6, 12, 16]. The Welsh-like siblings showed also low reductase activity in comparison to the Wistar rat and their heterozygous sister (Table 1). The low vitamin KO reductase activity has been associated with the enhanced (> 10-fold) dietary vitamin K requirement of the Welsh-derived homozygous warfarin-resistant rats [14, 17, 18]. In other words, the cellular vitamin K cycle does not meet the need for vitamin K hydroquinone. This means that warfarin resistance is not located at the site of vitamin KO reduction but has to be sought in the more efficient use of the dietary vitamin K. Apparently, the susceptible rat cannot use this vitamin K to overcome the block of the vitamin K cycle. The solution may lay in the dithiol-dependent microsomal reduction of vitamin K to vitamin K hydroquinone. The vitamin K reductase enzyme, whether or not identical with vitamin KO reductase [19, 20], shows the same reduced warfarin sensitivity [19]. Thus, also during warfarin exposure the Welsh-type resistant rat is provided by vitamin K hydroquinone from exogenous dietary vitamin K. In the susceptible rat, this pathway is blocked. Another way to provide the Welsh rat with the hydroquinone cofactor is via the warfarin-insensitive NADH-dependent microsomal vitamin K reduction. This activity has been shown to be enhanced in warfarin-resistant rats [6, 21]. Also the possibility that resistant rats may have acquired enhanced ability to synthesize vitamin K (i.e. menaquinone 4) from menadion (2-methyl-1,4-naphthoquinone, a vitamin additive used extensively on farms) [22] has to be considered.

The heterozygous rat, on the basis of its vitamin K (KO) reductase phenotype, is expected to be more susceptible to warfarin than the homozygote. However, the absence of the associated vitamin K deficiency syndrome (see discussion before) probably will be of favor as a selection criterium.

In summary, the results indicate the warfarin resistance of the particular wild rat to be of the Welsh resistant type. Both hetero- and homozygous individuals are found in the field.

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Inhibition of serum lactate dehydrogenase activity by disulfiram and diethyldithiocarbamate

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Treatment with oral disulfiram (DSF) is often prescribed as part of the medical management of alcohol abuse, with the objective of encouraging prolonged sobriety. Consumption of an alcoholic beverage during treatment with DSF will result in a subjectively unpleasant drug interaction characterized by malaise, flushing, palpitations and nausea [1, 2]. The mechanism of this interaction has been ascribed mainly to the non-competitive inhibition of aldehyde dehydrogenase by DSF, so that the consumption of ethanol is followed by toxic accumulation of its first metabolite, acetaldehyde [3, 4]. However, acetaldehyde toxicity does not completely account for all the clinical manifestations of the DSF–ethanol reaction, and the effects of the drug on other enzymes may also be clinically significant. DSF inhibits the activity of dopamine β-hydroxylase, which may contribute to the hypotension observed during the DSF–ethanol reaction [5]. Carper *et al.* have demonstrated recently that DSF and its major metabolite, diethyldithiocarbamate (DDC), both inhibit the activity of alcohol dehydrogenase. This unexpected new finding prompted us to investigate whether another major dehydrogenase enzyme, lactate dehydrogenase (LDH) (EC 1.1.1.27), was also inhibited by DSF.

Materials and methods

Preparation of DSF and DDC solutions. Disulfiram USP (Abbott Laboratories, North Chicago, IL) and diethyldithiocarbamate (Sigma Chemical Co., St Louis, MO) were dissolved in absolute alcohol (0.3 g DSF/100 ml and 0.17 g DDC/100 ml), stored at 2°, and used within 1 week of preparation.

Incubation procedure. Pooled human serum was prepared from blood samples submitted to the Department of Laboratory Medicine of St. Vincent's Medical Center of Richmond, and filtered through a Falcon sterile membrane filter unit No. 4620B10 (Thomas Scientific Co., Swedesboro, NJ). Duplicate 5-ml serum aliquots were incubated at 37°, and DSF or DDC solutions were added to produce 0, 50, 100, 200, 500, and 1000 μM concentrations. Samples of serum (0.5 ml) were withdrawn for assay from all incubation mixtures at zero time, and also at approximately 6, 12, 24 and 48 hr. The activity of LDH in each withdrawn sample was assayed by measuring the rate of formation of NADH in the reaction:

$$\text{lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

using a Beckman Ideal Clinical Analyzer (Beckman Instruments Inc., Brea, CA).

Results

The results of the incubations are shown in Fig. 1. Both DSF and DDC inhibited the activity of human serum LDH; the activity of LDH decreased in a log-linear fashion with time and also with the concentration of DSF and DDC. DSF appeared to be the more potent inhibitor of the enzyme, since there was no detectable activity after 24 hr of incubation with the 1000 μM solution.

Discussion

DSF and DDC both inhibited the activity of LDH in pooled human serum; the activity of the enzyme declined as the drug concentration and the duration of incubation were increased. The log-linear decline in enzyme activity with time suggests that this was a first-order process, i.e. that both DSF and DDC inactivated a constant fraction of the available enzyme per unit of time.

These findings raise a number of interesting questions which have yet to be answered. Since human serum contains at least five isoenzymes of LDH originating mainly from cardiac muscle, striated muscle, erythrocytes, liver, kidney, and brain, it is possible that not all the isoenzymes were equally susceptible to the drugs. In a clinical setting, reduced serum activity of LDH in patients treated with DSF or DDC could possibly obscure the diagnosis of acute