Assessment of the contribution of NAD(P)H-dependent quinone oxidoreductase 1 (NQO1) to the reduction of vitamin K in wild-type and NQO1-deficient mice

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INTRODUCTION

NQO1 [NAD(P)H quinone oxidoreductase 1; also known as DT-diaphorase] is a cytosolic enzyme that catalyses the two-electron reduction of various quinones including vitamin K. The enzyme may play a role in vitamin K metabolism by reducing vitamin K to vitamin K hydroquinone for utilization in the post-translational γ-glutamyl carboxylation reactions required by several proteins involved in blood coagulation. The aim of the present study was to assess the contribution of NQO1 to vitamin K reduction and haemostasis in an in vivo model. We examined the contribution of NQO1 to haemostasis by examining survival rates in mice poisoned with the anticoagulant warfarin. Supraphysiological amounts of vitamin K sufficiently reversed the effects of warfarin in both wild-type and NQO1-deficient mice.

Additionally, vitamin K reductase activities distinct from VKOR (vitamin K epoxide reductase) and NQO1 were measured in vitro from both wild-type and NQO1-deficient mice. The results of the present study suggest that NQO1 does not play a major role in vitamin K reduction during the production of vitamin K hydroquinone and supports the existence of multiple vitamin K reduction pathways. The properties of a NAD(P)H-dependent vitamin K reductase different from NQO1 are described.

Key words: γ-glutamyl carboxylase, NAD(P)H quinone oxidoreductase 1 (NQO1), NRH:quinone oxidoreductase 2 (NQO2), vitamin K, vitamin K epoxide reductase (VKOR), warfarin.

KH2 (vitamin K hydroquinone) is a cofactor for GGCX (γ-glutamyl carboxylase, EC 4.1.1.90), an enzyme that carboxylates certain glutamic acid residues of vitamin K-dependent proteins to Gla (γ-carboxyglutamic acid). This modification is essential in blood coagulation because it permits clotting factors such as Factors VII, IX and X to bind cofactors and cell surfaces, allowing the essential reactions of coagulation to occur [1]. With each carboxylation reaction, KH2 is oxidized to KO (vitamin K epoxide), a form of vitamin K that must be reduced by two separate two-electron reduction steps before further cycles of carboxylation can occur. The cyclic production of KO and its conversion back into KH2 constitutes the vitamin K cycle (Figure 1). GG CX and VKOR (vitamin K epoxide reductase, EC 1.1.4.1) are the only enzymes unequivocally identified as part of the vitamin K cycle [1].

In the vitamin K cycle, VKOR is responsible for reducing KO to vitamin K [2, 3] and it may also be involved in reducing vitamin K to KH2 [4–6]. Besides VKOR, however, there are other enzymes that contribute to vitamin K reduction. The most direct evidence for the presence of multiple vitamin K reduction enzymes arises from cases involving patients overdosed with the anticoagulant warfarin. Warfarin, which elicits its effects by antagonizing the ability of VKOR to reduce both KO and vitamin K, can be reversed by administration of large doses of vitamin K. It is presumed that, under these circumstances, vitamin K is reduced by an enzyme different from VKOR [7]. A model taking warfarin-resistant vitamin K reductase activities into account was first developed by Wallin et al. [8] who proposed that vitamin K reduction could be achieved by two independent pathways (Figure 1). Pathway I, as described by Wallin [8], consists of the thiol-dependent enzyme VKOR. Pathway II, on the other hand, consists of pyridine nucleotide-dependent enzymes that are less sensitive to anticoagulant drugs. The pathway II enzymes, therefore, are thought to be important in supporting vitamin K-dependent carboxylation reactions during warfarin poisoning [9]. The activities of the pathway II enzymes have been extensively characterized in vitro in several studies conducted by Wallin and co-workers [7,10,11], and their importance in sustaining vitamin K reduction in the absence of VKOR functionality was confirmed in a study conducted with VKOR-deficient mice. In a manner similar to warfarin-poisoned mice, VKOR-null mice die from bleeding unless they are supplemented with vitamin K [12].

The enzymes making up pathway II in Wallin’s model include NQO1 [NAD(P)H-dependent quinone oxidoreductase 1, EC 1.6.5.2], also known as DT-diaphorase, and an unidentified microsomal dehydrogenase(s) [9]. The existence of the two distinct enzymatic activities in the pathway was clearly established by Wallin et al. [10] who demonstrated that vitamin K could be reduced by NQO1 and a warfarin-resistant NAD(P)H-dependent enzyme present in microsomes immunodepleted of NQO1. The contribution of the two respective enzymatic activities to vitamin K metabolism during normal metabolism is unknown at this time. Because the gene encoding the warfarin-resistant microsomal dehydrogenase of pathway II is not known, it has not been possible to characterize its expression profile or enzymatic properties in a purified system. In contrast, much more is known about NQO1.

NQO1 is a ubiquitous dicoumarol-sensitive flavoprotein that can catalyse the two-electron reduction of several quinones including vitamin K (K1 and K2) (Figure 1) [13, 14]. The

Abbreviations used: ER, endoplasmic reticulum; GG CX, γ-glutamyl carboxylase; KH2, vitamin K hydroquinone; KO, vitamin K epoxide; NQO1, NAD(P)H quinone oxidoreductase 1; NQO2, NRH:quinone oxidoreductase 2; VKOR, vitamin K epoxide reductase; VKORL1, VKOR-like 1.

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potential importance of the role of NQO1 in supporting vitamin K-dependent protein carboxylation reactions was first suggested by Wallin et al. [15] who reported that rat liver microsomes depleted of NQO1 displayed reduced $\gamma$-glutamyl carboxylation activity. Although NQO1 was proposed to play a role in reducing vitamin K in some tissues, including rat liver, its overall importance in vitamin K reduction and haemostasis, if any, is unknown. On the basis of the low levels of NQO1 activity found in human liver cells, Wallin and Martin [16] proposed that NQO1 was only a minor component of pathway II in human liver tissue. This particular observation has led to the assumption that the unidentified microsomal dehydrogenase in pathway II of human liver cells is primarily responsible for overcoming warfarin toxicity. This hypothesis, however, has not been tested in vivo.

In the present study we studied NQO1-null mice to determine whether the enzyme plays an important role in the reduction of vitamin K needed for haemostasis when mice are poisoned with warfarin. Our results indicate that NQO1 is not required for haemostasis. On the other hand, we provide evidence that a warfarin-resistant NAD(P)H-dependent vitamin K reductase activity present in liver microsomes is sufficient for maintaining haemostasis during warfarin poisoning in mice. This enzyme is likely to be the same as that reported by Wallin, and may be responsible for this response.

EXPERIMENTAL

Reagents

NADH, NADPH, vitamin E acetate, BSA, dicoumarol, PMSF, warfarin, rat DT-diaphorase (catalogue number D1190; lot-specific number 120M1739V), an anti-NQO1 antibody, Mes, Tris, Bis-Tris, sodium acetate and CHAPS were obtained from Sigma–Aldrich. Vitamin K1 (10 mg/ml) was from Hospira. The pentapeptide substrate, FLEEL, was purchased from Bachem. NaH$^{14}$CO$_3$ was from ICN Pharmaceuticals. Acetonitrile, hexane, propan-2-ol and bicinechonic protein assay reagents were obtained from Thermo Fisher Scientific. PCR reagents were purchased from EMD and Stratagene. DTT was purchased from Research Products International. All other chemicals were reagent grade and were purchased from VWR International.

Experimental animals

The NQO1-deficient mice were previously characterized by Radjendirane et al. [17] and were obtained from the University of Maryland School of Medicine (Baltimore, MD, U.S.A.). For the experiment, 20 adult (20–25 g, aged 4–6 weeks) male NQO1-deficient mice crossed into C57BL/6 and 20 adult male wild-type mice (C57BL/6, Jackson Laboratories) were administered subcutaneous injections of warfarin twice daily at a concentration of 8 mg/100 g of body weight over 5 days. Half of the wild-type and NQO1-deficient mice receiving warfarin were also injected with vitamin K1 (Hospira) at a concentration of 13.6 mg/100 g of body weight two consecutive days before the administration of warfarin. The mice pre-treated with vitamin K1 were also supplemented with vitamin K at a concentration of 0.8 mg/100 g of body weight during warfarin administration. All mice were monitored daily for survival. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the University of North Carolina at Chapel Hill.

Preparation of liver microsomes and cytosol

Mouse livers were perfused and washed with an ice-cold saline solution before excision. The livers were homogenized in 50 mM Mes (pH 6.5) and 1 mM PMSF. The homogenate was centrifuged at 12000 rev./min for 10 min in a Beckman Type 70Ti rotor at 4°C. The supernatant was recovered and centrifuged in the same rotor at 40000 rev./min for 60 min at 4°C. The soluble
portion was collected as the cytosol and the microsomal pellet was resuspended in 50 mM Mes (pH 6.5) and 1 mM PMSF at a protein concentration of 5–10 mg/ml and stored at −80°C.

**Preparation of KH2**

KH2 was synthesized by chemically reducing vitamin K1 with DTT as described previously [18]. Vitamin K1 was incubated at 37°C overnight in a solution containing 25 mM Tris (pH 8.5), 0.5 M NaCl and 0.2 M DTT. The concentrations of vitamin K1 and reduced vitamin K1 were determined spectrophotometrically in 95% ethanol using the molar absorption coefficients 18209 M⁻¹·cm⁻¹ at 248 nm for vitamin K1 and 48154 M⁻¹·cm⁻¹ at 244 nm for reduced vitamin K1 [19].

**In vitro NQO1 activity assays and Western blot analysis**

NQO1 activity was measured as described previously [20]. The reaction mixture in a final volume of 200 μl contained 25 mM Tris/HCl (pH 7.5), 0.01% Tween 20, 0.7 mg/ml BSA, 5 μM FAD, 200 μM NADH, 40 μM menadione or 180 μM vitamin K1, and recombinant rat NQO1 or mouse liver cytosol. The decrease in NADH absorbance at 340 nm was followed using a Spectra Max M5 spectrophotometer (Molecular Devices). Expression levels of NQO1 were analysed by SDS/PAGE and immunoblotting with anti-NQO1 antibodies. Blots were developed with ECL reagents.

**γ-Glutamyl carboxylation activity assays**

Vitamin K1 reductase activity was assessed using a coupled assay measuring GGCX activity. Endogenous GGCX activity was determined by following the incorporation of 14C into the pentapeptide substrate FLEEL [18]. Standard assay conditions contained 50 mM Mes (pH 6.5), 200 μM warfarin, 3.6 mM FLEEL, 4 μM Factor IX propeptide, 5 mM DTT, 5 mM MnCl2, 1.0% CHAPS, NaHCO3 (120 μCi/ml) and 5 mg/ml enzyme. Reactions were initiated with 440 μM vitamin K and 2 mM NADH or with 180 μM chemically prepared KH2 and incubated for the indicated times at 20°C. The amount of 14CO2 incorporation was determined as described previously [18].

**Assay of warfarin-resistant vitamin K reductase activity**

Warfarin-resistant vitamin K reductase activity was measured in 200 μl reaction mixtures containing 50 mM Mes (pH 6.5), 440 μM vitamin K1, 1% CHAPS, 200 μM warfarin, 2 mM NADH, 5 mM MnCl2, 5 mM DTT and 0.5–5 mg/ml microsomes, or under the conditions described in the text. The reaction mixtures were incubated in the dark at 30°C for the times indicated, and the reactions were stopped by adding 500 μl of propan-2-ol and 500 μl of hexane containing 15 μg/ml vitamin E acetate as an internal standard. The vitamin K metabolites were extracted into the upper hexane phase by mixing and centrifugation. Samples were centrifuged at 25°C for 2 min at 12000 g. A 500 μl aliquot of the upper hexane phase was removed and evaporated to dryness under nitrogen at room temperature (25°C). The residue was dissolved in 300 μl of HPLC mobile-phase buffer [acetonitrile/propan-2-ol/water, 100:7:2 (by vol.)] and 100 μl of the sample volume was analysed by RP-HPLC (Agilent 1100, Agilent Technologies) using a C18 column (Waters; column particle size 4 μm, column dimensions 3.9 mm×300 mm). The absorbance of the eluting material was measured at 248 nm with diode array detection. The eluted material was also monitored with flow-cell fluorimetry (λex = 246 nm and λem = 430 nm). The retention time for vitamin K1 and reduced vitamin K1 were 6.6 and 19.0 min respectively at a flow rate of 1.5 ml/min. Quantification was based on the integration of the fluorescent peaks compared with peaks from standards of freshly prepared and quantified reduced vitamin K1.

**Apparent kinetic parameters of warfarin-resistant vitamin K reductase activity**

Velocity as a function of vitamin K1 concentration was determined using optimized assay conditions, except that NADH was held constant at 2 mM and vitamin K1 was varied from 1.7 to 440 μM. The velocities were fit to the Michaelis–Menten equation using KaleidaGraph. The apparent kinetic parameters with respect to NADH and NADPH were determined as described above, except that vitamin K was held constant at 440 μM, whereas NADH and NADPH were varied from 0.05 to 4 mM.

**Effect of pH and detergent on warfarin-resistant vitamin K reductase activity**

The pH-dependency of the warfarin-resistant vitamin K reductase activity was determined by assaying liver microsomes in a
Figure 3 Survival rate of wild-type and NQO1-deficient mice challenged with warfarin

Wild-type (A) and NQO1-deficient mice (B) received vitamin K1 (13 mg/100 g of body weight) or no vitamin K1 for 2 days before administration of warfarin. Beginning on day 0, all mice were given warfarin twice daily at a concentration of 8 mg/100 g of body weight. The mice pre-treated with vitamin K1 continued to receive vitamin K1 during warfarin administration at a dose of 0.8 mg/100 g of body weight. The arrow depicts the start of administration of warfarin. In each experimental group, ten mice were used.

three-component buffer system consisting of 100 mM sodium acetate, 50 mM bis(2-hydroxymethyl)-imino-tris(hydroxy-methyl)-hexane and 50 mM Tris across a pH range of 5–9 as described previously [21]. Standard assay conditions were used, except that the usual buffer [50 mM Mes (pH 6.5)] was replaced with the triple buffer. The detergent-dependency of the warfarin-resistant vitamin K reductase activity was determined by assaying liver microsomes under standard conditions, except that CHAPS was varied from 0 to 64 mM.

RESULTS

Analysis of NQO1-deficient mice

The levels and activities of NQO1 in wild-type and NQO1-deficient mice were examined by Western blotting and enzymatic analysis. The cytosolic and microsomal liver fractions from wild-type and NQO1-deficient mice were immunoblotted with an antibody that reacts with human, rat and mouse NQO1 (Figure 2). The antibody reacted with purified rat NQO1 (lane 1), as well as with NQO1 present in mouse liver cytosolic fractions. As shown in lane 3 of Figure 2, the cytosolic fraction from the NQO1-deficient mouse lacked the NQO1 protein that is present as a 32 kDa band in the wild-type cytosol fraction (lane 2). The NQO1 protein was also absent in mouse liver microsomes (lanes 4 and 5). A smaller cross-reacting protein, however, was present in both the wild-type and NQO1-deficient mouse microsome samples.

NQO1 activity is typically measured with menadione (K3), a chemically synthesized derivative of vitamin K. The naturally occurring forms of vitamin K (K1 and K2) (Figure 1), however, are reported to be poor substrates for NQO1 [6,14]. We confirmed this by analysing the menadione and vitamin K1 reductase activities of purified NQO1 (Table 1). The rates of vitamin K1 reduction were negligible relative to that of menadione. As expected, dicoumarol was a potent inhibitor of the activity observed with menadione. No significant statistical differences, however, were observed in the rate of vitamin K1 reduction in the presence of the inhibitor. Next we measured NQO1 activity in the presence and absence of dicoumarol in the wild-type and NQO1-deficient mice liver preparations (Table 1). The dicoumarol-sensitive activity of the liver cytosolic fractions was interpreted as NQO1 activity. NQO1 activity was absent in the NQO1-deficient mouse liver cytosol when assaying with menadione and no detectable activity was observed with vitamin K1. Because the kinetic properties of NQO1 can be strongly influenced by the assay conditions used [22], it is worth noting that the results presented here are in agreement with previous studies [6,13,17,23].

Effect of vitamin K and warfarin on NQO1-deficient mice

In order to test the contribution and importance of NQO1 to the vitamin K cycle, survival rates were monitored in wild-type and NQO1-deficient mice poisoned with warfarin, a condition in which VKOR function is compromised. Age-matched wild-type and NQO1-deficient male mice were administered warfarin with or without vitamin K supplementation. Both the wild-type and the knockout mice treated with vitamin K survived (Figure 3). All NQO1-deficient mice under these experimental conditions were able to produce reduced vitamin K by a mechanism independent of VKOR and NQO1. In contrast, all mice lacking vitamin K supplementation failed to survive past 5 days due to bleeding.
NQO1 is not essential for haemostasis in warfarin-poisoned mice

Vitamin K reductase activity was measured under standard assay conditions (440 μM vitamin K1, 200 μM warfarin, 2 mM NADH, 1 mM MnCl2, 5 mM DTT, 1% CHAPS and 1 mg/ml NQO1-deficient mouse liver microsomes in 50 mM Mes (pH 6.5)) or under the conditions specified. Results are relative to those obtained using standard reaction conditions. All reactions were carried out at 30°C for 30 min. 1, Standard reaction mix; 2, standard assay conditions with 2 mM NADPH replacing 2 mM NADH; 3, standard assay conditions without NADH; 4, standard assay conditions without warfarin; 5, standard assay conditions without MnCl2; 6, standard assay conditions without DTT; and 7, standard assay conditions with boiled enzyme.

Vitamin K reductase activity of microsomes isolated from wild-type and NQO1-deficient mice

It has been established previously that there are at least two pathways responsible for the reduction of vitamin K: a VKOR-mediated pathway, and a second pathway mediated by an enzyme independent of VKOR [9,11]. In order to gain insight into this process, as well as to identify the enzymatic activities that may play a role in rescuing the warfarin-poisoned NQO1-deficient mice, mouse liver microsomes were analysed for warfarin-resistant vitamin K reductase activity. Vitamin K reduction was measured using a coupled γ-glutamyl carboxylation assay (Table 2). In this assay, the GGCX substrate FLEEL is carboxylated when reduced vitamin K1 is available. The GGCX activity in the reactions was supported by supplying chemically generated KH2 directly or by supplying vitamin K and a nicotinamide cofactor [9]. An NADH-dependent vitamin K reductase activity was observed in both wild-type and NQO1-deficient mice. Carboxylase activity in the presence of vitamin K and NADH resulted in a rate of carboxylation that was comparable with that generated by supplying chemically reduced KH2 directly. In the absence of NADH, very little carboxylation of the substrate was observed.

Development of a direct assay for the detection of vitamin K reductase activity

In an attempt to characterize the warfarin-resistant vitamin K reductase activity present in mouse liver microsomes in a more specific manner, we developed and optimized an RP-HPLC assay method for the direct detection of KH2. The necessary components for observing vitamin K1 reductase activity in the assay were determined by varying the components used in the carboxylation reaction (Figure 4). Consistent with the carboxylation reaction, reduced vitamin K accumulated only when a nicotinamide cofactor (NADH or NADPH) was present (Figure 4, columns 1–3). The amount of KH2 detected in the absence of warfarin was only slightly higher relative to that carried out in the presence of 200 μM warfarin (Figure 4, column 4). This result indicates that the observed activity under the experimental conditions used is independent of VKOR. Intriguingly, the metal manganese, which has been commonly used in carboxylation assays [11], was also found to be important for activity (Figure 4, column 5). The reductant DTT was also required to observe hydroquinone accumulation (Figure 4, column 6). The reductant may be required to stabilize any KH2 that is formed. DTT can reduce vitamin K directly, but under the experimental assay conditions used in the present study, the amount of KH2 accumulating in reactions lacking enzyme was less than 10% relative to those containing enzyme. In all of the experiments described herein, the KH2 formed non-enzymatically in reactions containing DTT has been taken into account and subtracted. To confirm that an enzyme was responsible for vitamin K hydroquinone accumulation, an assay was conducted with mouse liver microsomes that had been boiled for 10 min (Figure 4, column 7). The loss of activity in this reaction confirms the presence of an NADH-dependent enzyme. When the reactions contained the relevant components, reactions progressed linearly for at least 30 min and were also linear with protein concentration (Figure 5). There were no significant differences in the specific activities of the vitamin K reductase activities.
Figure 6 Effect of vitamin K1 and nucleotide cofactor concentrations on vitamin K1 reductase activity of NQO1-deficient mouse liver microsomes

(A) Vitamin K1 reductase activity was assayed under standard conditions except that vitamin K1 was varied from 27 to 440 μM. (B) Vitamin K1 was held constant at 440 μM and NADH was varied from 50 μM to 4 mM. (C) Vitamin K1 was held constant at 440 μM and NADPH was varied from 50 μM to 4 mM.

from wild-type and NQO1-deficient mice (wild-type microsomes, 1.71 ± 0.05 nmol/min per mg; NQO1-deficient microsomes, 2.01 ± 0.07 nmol/min per mg). The NQO1-deficient mouse liver microsomes were thus used for further analysis.

Apparent kinetic parameters for the warfarin-resistant vitamin K reductase activity

The kinetic properties of the observed warfarin-resistant vitamin K1 reductase activity present in NQO1-deficient mouse livers was characterized by examining activity in the presence of various amounts of vitamin K1 or reduced dinucleotide cofactor. Enzymatic activity was found to be dependent on vitamin K1 concentration and a Michaelis–Menten-type relationship was observed (Figure 6A). The apparent $K_m$ value calculated from a Michaelis–Menten function was 174 μM.

The effects of reduced dinucleotide concentrations on enzymatic vitamin K1 reduction were also investigated by varying the concentration of the two nucleotides between 50 μM and 4 mM. Vitamin K1 reductase activity was stimulated by increasing concentrations of the reduced dinucleotides NADH and NADPH (Figure 6B). A reduction in activity was observed for both reduced dinucleotides at high concentrations. This result demonstrates that the warfarin-resistant vitamin K reductase(s) responsible for the relevant activity, like NQO1, can use either nucleotide. Thus the unidentified enzyme(s) characterized here could potentially have contributed to activities previously assigned to NQO1 in crude preparations.

Effect of detergent and pH on vitamin K reductase activity

The detergent-dependency of the warfarin-sensitive vitamin K reductase activity was determined by varying the amount of CHAPS in the assay (Figure 7A). Enzymatic activity was not detected when intact microsomes were used as the enzyme source. Activity, however, was stimulated when the concentration of CHAPS exceeded its CMC (critical micelle concentration) value (10 mM). These properties are consistent with the proposed ER (endoplasmic reticulum) location of the enzyme.

The optimal pH for enzymatic activity was also determined by varying the pH in a triple-buffer reaction solution. Optimal activity was detected at neutral pH values. These results should prove useful in identification and characterization of the unidentified vitamin K reductase(s).

DISCUSSION

Vitamin K reductase activities independent of VKOR have often been credited to NQO1 [1,12,24,25]. Since its discovery, it has been speculated that NQO1 played a role in vitamin K metabolism. The activities associated with NQO1 were initially thought to be important in both vitamin K reduction and mitochondrial electron transport, and the enzyme was given the name vitamin K reductase [26]. With the discovery of the vitamin K cycle, NQO1 was later proposed to be involved in the vitamin K recycling reactions of the vitamin K cycle [15]. In support of this hypothesis, purified preparations of NQO1 were shown to be capable of reducing vitamin K1, albeit with substantially lower efficiency relative to the vitamin K analogue menadione [6,13]. Additionally, vitamin K1 reduction mediated by NQO1 was found to be insensitive to warfarin, lending support to the idea that the enzyme could contribute to haemostasis when patients were overdosed with warfarin [13]. There are, however, several reasons why NQO1 may not play a major role in the vitamin K cycle and haemostasis. First, as reported by Wallin and Hutson [10], there appears to be another warfarin-resistant enzyme that can reduce vitamin K under conditions of warfarin toxicity. Next, NQO1 is a cytoplasmic enzyme and is physically separated from the microsomal enzymes involved in the vitamin K cycle. Although microsomal NQO1 activity has been described previously, a role for the microsomal-associated NQO1 activity in vitamin K reduction has not been established [27]. Additionally, there are no reported correlations between NQO1 polymorphisms and thrombosis risk [28]. Lastly, the NQO1-deficient animals
previously described and used in the present study display no general bleeding problems [17].

Because of its history of being associated with vitamin K metabolism and proposed function, we felt it was important to directly determine whether NQO1 played any significant role in vitamin K reduction in an in vivo model. To accomplish this, we analysed the ability of vitamin K to act as an antidote when warfarin was administered to NQO1-deficient mice. We found that NQO1-deficient mice receiving both vitamin K1 and warfarin survived at the same frequency as the wild-type mice. Our results are in agreement with a study by Wallin and Hutson [10] in which residual warfarin-resistant vitamin K reductase activity remained when antibodies against NQO1 were used to neutralize the enzyme in solubilized rat microsomes. The present study, however, is the first to show that NQO1 does not play a vital role in vitamin K-mediated haemostasis. NQO1 may play some role in vitamin K metabolism, but it clearly is not required for haemostatic functions when warfarin is present.

The results of the present study support the hypothesis that there are multiple enzymes that reduce vitamin K1. Besides VKOR and NQO1, other known possible candidate vitamin K1 reductases include enzymes with sequences and function similar to those of NQO1 and VKOR: NQO2 (NRH:quinone oxidoreductase 2, EC 1.10.99.2) and VKORL1 (vitamin K epoxide reductase-like 1). NQO2, like NQO1, is a cytosolic flavoprotein that is also believed to be involved in metabolic detoxification of quinones. NQO2, however, does not use phosphorylated nicotinamides [NAD(P)H] as reductants. Instead, NQO2 utilizes non-phosphorylated nicotinamide derivatives [14]. Further research is needed to investigate the importance, if any, of this enzyme in the vitamin K cycle. We, however, believe that an enzyme different from NQO2 may be involved in the reduction of vitamin K when the NQO1 mice are poisoned with warfarin. The enzyme responsible for reducing vitamin K in our in vitro analysis clearly is not NQO2 because NADH or NADPH is required for vitamin K1 reduction. The utilization of pyridine nucleotide cofactors is also inconsistent with the properties of the other candidate, VKORL1, a parologue of VKOR. VKORL1 is an intriguing candidate vitamin K reductase because it is in the ER and is capable of reducing both vitamin K epoxide and vitamin K [29]. Less is known about VKORL1 than about VKOR, but the enzyme is currently proposed to play a role in vitamin K-mediated intracellular anti-oxidation pathways. The enzyme may reduce vitamin K under normal physiological conditions, but it most likely is not responsible for rescuing the warfarin-poisoned mice since the enzyme is also susceptible to warfarin [29].

On the basis of that conclusion then, what is the enzyme of importance in the vitamin K cycle? To start answering that question, we confirmed that there is a warfarin-resistant vitamin K reductase present in both wild-type and NQO1-deficient mice. The clinical relevance of the vitamin K reductase activities described in the present study, however, is still uncertain. Our results, however, illustrate the need for identifying the other enzymes contributing to vitamin K metabolism. The assay developed in the present study should facilitate future purification attempts.

AUTHOR CONTRIBUTION

Brian Ingram and Darrel Stafford contributed to the study design and conception. Data acquisition was performed by Brian Ingram, Jared Turbyfill and Peggy Bledsoe. Anil Jaiswal provided reagents and critical advice during preparation of the paper. Brian Ingram and Darrel Stafford were involved in the analysis and interpretation of data. Brian Ingram wrote the paper. Darrel Stafford revised the paper.

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