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Tris(3-hydroxypropyl)phosphine is superior to dithiothreitol for *in vitro* assessment of vitamin K 2,3-epoxide reductase activity

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ABSTRACT

Use of the reductant dithiothreitol (DTT) as a substrate for measuring vitamin K 2,3-epoxide reductase (VKOR) activity *in vitro* has been reported to be problematic because it enables side reactions involving the vitamin K_1 2,3-epoxide (K_1>O) substrate. Here we characterize specific problems when using DTT and show that tris(3-hydroxypropyl)phosphine (THPP) is a reliable alternative to DTT for *in vitro* assessment of VKOR enzymatic activity. In addition, the pH buffering compound imidazole was found to be problematic in enhancing DTT-dependent non-enzymatic side reactions. Using THPP and phosphate-based pH buffering, we measured apparent Michaelis–Menten constants of 1.20 μM for K_1>O and 260 μM for the active neutral form of THPP. The K_m value for K_1>O is in agreement with the value that we previously obtained using DTT (1.24 μM). Using THPP, we successfully eliminated non-enzymatic production of 3-hydroxyvitamin K_1 and its previously reported base-catalyzed conversion to K_1, both of which were shown to occur when DTT and imidazole are used as the reductant and pH buffer, respectively, in the *in vitro* VKOR assay. Accordingly, substitution of THPP for DTT in the *in vitro* VKOR assay will ensure more accurate enzymatic measurements and assessment of warfarin and other 4-hydroxycoumarin inhibition constants.

Vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1)[2] is the rate-limiting enzyme for the activation of vitamin K-dependent (VKD) blood clotting proteins and the molecular target of warfarin and other 3-hydroxycoumarin oral anticoagulants [1–3]. *In vivo*, VKORC1 transfers reducing equivalents via disulfide exchange reactions from endoplasmic reticulum (ER) lumenal oxidoreductase enzymes, reduced by catalyzing intramolecular disulfide bond formation during de novo oxidative protein folding, to vitamin K_1 2,3-epoxide (K_1>O) or to vitamin K_1 (K_1) that becomes reduced to K_1 or vitamin K_1 hydroquinone (K_1H_2), respectively. This enables recycling of limited intracellular amounts of K vitamers to drive multiple rounds of the vitamin K redox cycle.

Because the identity (identities) of the physiological partner oxidoreductase that reduces VKORC1 to enable vitamin K 2,3-epoxide reductase (VKOR) and vitamin K reductase (VKR) activities is not known, low molecular weight mono- and dithiol-containing compounds, including cysteine, reduced glutathione, lipoamide, reduced lipoic acid, β-mercaptoethanol, 1,2-ethanedithiol, 1,4-butanedithiol, dithioerythritol (DTE), and dithiothreitol (DTT), have historically been used as substrates to drive the VKOR enzymatic reaction for *in vitro* studies [4,5]. In 1978, Whitlon and coworkers established that DTT and vitamin K_1 2,3-epoxide substrates could sustain robust VKOR activity in rat microsomal preparations [6]. Subsequently, all published studies that measured *in vitro* VKOR activity have consistently used DTT as the reducing substrate (see, e.g., Refs. [7–10]). Since the identification of the human and rodent VKORC1 genes and enzymes in 2004 [1,2], a steadily

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[2] *Abbreviations used:* VKORC1, vitamin K 2,3-epoxide reductase complex subunit 1; VKD, vitamin K-dependent; ER, endoplasmic reticulum; K_1>O, vitamin K_1 2,3-epoxide; K_1, vitamin K_1 (phylloquinone); K_1H_2, vitamin K_1 hydroquinone; VKOR, vitamin K 2,3-epoxide reductase; VKR, vitamin K reductase; DTE, 1,4-butanedithiol dithioerythritol; DTT, dithiothreitol; 3OH-K_1, 3-hydroxyvitamin K_1; UV, ultraviolet; TBP, tris(*n*-butyl)phosphine; THPP, tris(3-hydroxypropyl)phosphine; TCEP, tris(2-carboxyethyl)phosphine; THMP, tris(hydroxymethyl)phosphine; PBS7.4, phosphate-buffered saline pH 7.4; EGFP, enhanced green fluorescent protein; β2AR, β2-adrenergic receptor; HPLC, high-performance liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate.

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growing number of published reports have used the DTT-driven assay to study VKOR enzymatics and inhibition by warfarin (see online Supplementary material for a list of literature references).

Production of a covalent 2-thiol-3-hydroxy DTT adduct of K_1 and an isomer of 3-hydroxyvitamin K_1 (3OH-K_1) as minor products in DTT-driven VKOR assay samples was first reported by Hildebrandt and coworkers in 1984 based on previous characterization of these compounds by Preusch and Suttie [10,11]. The DTT adduct was identified by ultraviolet (UV), infrared (IR), and ^{1}H nuclear magnetic resonance (NMR) spectroscopic analyses as the product of direct nucleophilic attack of DTT thiolate at the oxirane ring of K_1>O that results in conversion of the epoxide to a *cis*-hydroxy group. Resolution of the DTT adduct by a second nucleophilic thiolate attack results in 3OH-K_1. Furthermore, triethylamine alone was previously shown to be capable of catalyzing the removal of water from 3OH-K_1 by a general base catalysis mechanism to yield the quinone form of vitamin K_1 [11].

Tris(alkyl)phosphines and derivative compounds efficiently and stoichiometrically reduce cystine disulfides by a hydrolysis-mediated mechanism that results in two cysteines and the respective tris(alkyl)phosphine oxide [12]. The reaction is essentially irreversible and, unlike DTT, is not capable of mediating direct nucleophilic attack on K_1>O. In general, tris(alkyl)phosphines are more stable to air oxidation than DTT [13]. In 2003, Wallin and coworkers first reported the use of organophosphine compounds, in lieu of DTT, as reductant substrates for studying VKOR activity [14]. They showed that tris(*n*-butyl)phosphine (TBP) was slightly more effective than DTT as a reducing substrate for driving the VKOR reaction in rat liver microsomes. Subsequent studies by Chu and coworkers and Jin and coworkers relied on a similar reductant, tris(3-hydroxypropyl)phosphine (THPP), for maintaining the enzyme in a reduced state during purification [15,16]. However, both studies used DTT as the chief reductant for assessment of VKOR activity. Here we show that substitution of THPP for DTT and substitution of phosphate for imidazole buffer in the *in vitro* VKOR assay eliminates the non-enzymatic conversion of K_1>O to 3OH-K_1 described in earlier reports [10,11].

Materials and methods

Chemicals and reagents

All biochemical reagents were purchased or prepared and concentrations determined as described previously [17,18]. In addition, THPP and tris(2-carboxyethyl)phosphine (TCEP) were obtained from Calbiochem (EMD Biosciences, Darmstadt, Germany), and tris(hydroxymethyl)phosphine (THMP) was obtained from Acros Organics (Fisher Scientific, Nidderau, Germany). Concentrated stock solutions of TCEP were adjusted to pH 7.4 in phosphate-buffered saline before addition to assay samples. Phosphate-buffered saline pH 7.4 (PBS7.4, Sigma–Aldrich Chemie, Munich, Germany) is composed of 0.01 M phosphate-buffered saline (0.138 M NaCl and 0.0027 M KCl) at pH 7.4 and 25 °C. Buffer B (the standard buffer used in previously published *in vitro* studies of VKOR enzymatic activity) was composed of 25 mM imidazole (pH 7.6) and 0.5% (w/v) CHAPS.

Production of human VKORC1 in Pichia pastoris

Chimeric human VKORC1–EGFP (enhanced green fluorescent protein) was produced and enriched in ER membranes as described previously [17]. As negative control, we used ER-enriched *Pichia* membranes with a similar EGFP chimera constructed using the human β2-adrenergic receptor (β2AR–EGFP).

In vitro DTT- or organophosphine-driven VKOR assays

Standardized VKOR assay samples contained (added in order listed) 495 µl of buffer B or PBS7.4 with 0.5% CHAPS, 10 µl of *Pichia* ER-enriched membranes (166.6 µg dry mass) containing either 250 ng of expressed VKORC1 or approximately 250 ng of β2AR–EGFP in storage buffer, 10 µl of 20 mM warfarin in ethanol (final concentration in assay 377 µM; no ethanol added for samples without warfarin), 10 µl of K_1>O at various concentrations in ethanol, 20 µl of either 125 mM DTT dissolved in buffer B (for the standard DTT-driven assay) or various concentrations of DTT, THPP, THMP, or TCEP in PBS7.4 with 0.5% CHAPS as indicated in Results and figure legends. Assay samples were briefly mixed by vortexing after the addition of each component. Assay method was as described previously [17].

HPLC and spectrophotometric analysis of VKOR reaction products

Sample separation and analysis were performed as described previously [17]. Chemical identities of eluted quinone peaks were confirmed by comparison of diode array detector (DAD)-obtained UV spectra with those from published authenticated standards from the literature (K_1, high-performance liquid chromatography [HPLC] elution time 10.35–10.36 min [19]; K_1>O, 7.76–7.77 min [20]; 3OH-K_1, 5.68–5.69 min [21,22]; Q_8, 8.12–8.15 min [23]). Initial velocities were calculated as K_1 product normalized to the relative amount of enzyme in each sample as described previously [17]. Slopes for Eisenthal and Cornish-Bowden direct linear plot analysis were calculated as initial velocity divided by initial K_1>O substrate concentration (see Supplementary material for details).

Results

Non-enzymatic reduction of K_1>O in the DTT-driven VKOR assay is enhanced by general base catalysis

Based on previous reports of non-enzymatic reaction artifacts for the *in vitro* DTT-driven VKOR enzymatic assay [10], we first assessed contributing factors for samples lacking VKORC1 using a recently standardized version of the assay (Fig. 1) [17,18]. For all pH-buffered and -unbuffered systems we investigated, incubation of K_1>O for 1 h without reductant showed no reaction (Fig. 1; compare left-most gray-colored 100% bars for each solution/buffer condition). When we added DTT (final concentration 5 mM) to each solution/buffer containing K_1>O for the 1-h assay, K_1>O conversion to 3OH-K_1 was measured for all conditions (Fig. 1, white bar segments in the middle bar stacks marked "… +DTT" for the left-most four solution/buffer groups). Specifically, for unbuffered water there was 21 ± 7.2% conversion to 3OH-K_1, consistent with a reported pH range of 4.0 to 6.0 for DTT dissolved in unbuffered water where only 0.07 to 0.0007% of total DTT is the active thiolate form [24]. For imidazole-containing buffers at pH 7.6, where the Henderson–Hasselbalch equation predicts that 2.8% of total DTT (pK_{a1} 9.2, pK_{a2} 10.1) is the active thiolate form, there was a concentration-dependent increased production of 3OH-K_1 of 25.6 ± 8.4% for 25 mM imidazole and 50 ± 14.1% for 500 mM imidazole (white bar segments). Small quantities of K_1 (Fig. 1, black bar segments in the middle bar stacks marked "… +DTT" for the left-most four solution/buffer groups) were also generated in the presence of DTT and imidazole (2.8 ± 2.8% for 25 mM imidazole, 3 ± 2.5% for 500 mM imidazole). When we substituted PBS7.4 for imidazole in the presence of DTT, there was a dramatic decrease of non-enzymatic 3OH-K_1 and K_1 products relative to the amounts for imidazole buffer (Fig. 1, middle bar stack marked "… +DTT" for the fourth solution/buffer group; 15 ± 2.8% 3OH-K_1, 1 ± 0.5% K_1).