HUMAN VITAMIN K 2,3-EPoxide REDUCTASE COMPLEX SUBUNIT 1-LIKE 1 (VKORC1L1) MEDIATES VITAMIN K-DEPENDENT INTRACELLULAR ANTIOXIDANT FUNCTION* 

Philipp Westhofen1,‡, Matthias Watzka1,§, Milka Marinova1, Moritz Hass1, Gregor Kirfel2, Jens Müller1, Carville G. Bevans1, Clemens R. Müller1, Johannes Oldenburg§

From Institute of Experimental Haematology and Transfusion Medicine, Sigmund-Freud-Strasse 25, University Clinic Bonn, 53127 Bonn, Germany1, Institute for Cell Biology, Ulrich-Haberland-Strasse 61a, University Bonn, 53120 Bonn, Germany2, and Institute of Human Genetics, University of Würzburg, Biozentrum Am Hubland, 97074 Würzburg, Germany3

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Address correspondence to: Prof. Dr. med. Johannes Oldenburg, Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Sigmund-Freud-Str. 25, Bonn, Germany. Phone: +49 228 287 15175; Fax: +49 228 287 14783; Email: johannes.oldenburg@ukb.uni-bonn.de

Human vitamin K 2,3-epoxide reductase complex subunit 1-like 1 (VKORC1L1), expressed in HEK 293T cells and localized exclusively to membranes of the endoplasmic reticulum, was found to support both vitamin K 2,3-epoxide reductase (VKOR) and vitamin K reductase enzymatic activities. Michaelis-Menten kinetic parameters for dithiothreitol-driven VKOR activity were $K_m$ (μM) = 4.15 (vitamin K$_1$ epoxide), 11.24 (vitamin K$_2$ epoxide); $V_{max}$ (nmol mg$^{-1}$ hr$^{-1}$) = 2.57 (vitamin K$_1$ epoxide), 13.46 (vitamin K$_2$ epoxide). Oxidative stress induced by H$_2$O$_2$ applied to cultured cells upregulated VKORC1L1 expression and VKOR activity. Cell viability under conditions of no induced oxidative stress was increased by the presence of vitamin K$_1$ and vitamin K$_2$, but not ubiquinone-10, and was specifically dependent on VKORC1L1 expression. Intracellular reactive oxygen species levels in cells treated with 2,3-dimethoxy-1,4-naphthoquinone were mitigated in a VKORC1L1 expression-dependent manner. Intracellular oxidative damage to membrane intrinsic proteins was inversely dependent on VKORC1L1 expression and the presence of vitamin K$_1$. Taken together, our results suggest that VKORC1L1 is responsible for driving vitamin K-mediated intracellular antioxidation pathways critical to cell survival.

Aging and age-related chronic diseases including Alzheimer disease, amyotrophic lateral sclerosis, atherosclerosis, cancer, diabetes, Parkinson disease, and rheumatoid arthritis, among others, are widely recognized as resulting from cellular damage inflicted by reactive oxygen species (ROS)†(1), yet cells produce ROS as a result of normal aerobic metabolism including oxidative phosphorylation in mitochondria and oxidative protein folding (OPF) in the endoplasmic reticulum (ER). Intracellular antioxidant defence systems, evolved to combat ROS-induced damage, include direct enzymatic scavengers (e.g., superoxide dismutases, peroxidases) and non-enzymatic small molecule antioxidants (e.g., vitamins C, E and glutathione) that are maintained in their active, reduced forms by antioxidant enzymatic scavengers(2). Recently, vitamin K hydroquinone (KH$_2$) was recognized as a potent biological antioxidant(3,4), but regenerative antioxidative enzymatic mechanisms have not been identified for this required trace nutrient.

In vertebrates, K vitamins and their 2,3-epoxides are recognized substrates of vitamin K 2,3-epoxide reductase complex subunit 1 (VKOR1), an enzyme required for blood clotting and bone homeostasis(5). Recently, the first high-resolution structure of a prokaryotic vitamin K epoxide reductase (VKOR) enzyme from the fully sequenced thermophilic cyanobacterial Synechococcus species JA-2-3’a(2-13) (for which the VKOR enzyme will be subsequently identified as
"proVKOR" in this article(6,7) was solved to 3.6 Å resolution and is responsible for passing reducing equivalents from de novo OPF in the periplasmic space to lipidic quinones in the cell outer membrane(8). In contrast to genomes of archaea, euubacteria, plants, protists and lower animals that include a single VKOR protein ortholog, vertebrate genomes include two paralogous enzymes, VKORC1 and VKORC1-like 1 (VKORC1L1), likely resulting from a gene duplication of an early common VKOR ancestor(9-12). Although the function and subcellular location of VKORC1 were well characterized already two decades before identification of the gene(13), there has been no informative study of function or location for VKORC1L1.

Here we show that VKORC1L1 is responsible for vitamin K-mediated increased survival of oxidatively stressed cells, is responsible for limiting the amount of intracellular ROS, and plays a key role in mitigating oxidative damage to membrane proteins. Thus, VKORC1L1 apparently plays a ubiquitous, fundamental role in intracellular antioxidation.

EXPERIMENTAL PROCEDURES

Cell culture and transfection- HEK 293T human embryonic kidney cells (ATCC cell line CRL-11268) were cultured in MEM, 10% FBS in a humidified atmosphere, 5% CO2, 37°C. Cells 80-90% confluent were transfected with pcep4 (Invitrogen, Darmstadt, Germany) mammalian expression vector constructs with integrated cDNA open reading frames encoding human VKORC1L1, VKORC1 or γ-glutamyl carboxylase (GGCX) proteins using Fugene HD transfection reagent (Roche, Grenzach-Wyhlen, Germany) according to the manufacturer's instructions. For VKORC1L1 transcription knock-down, 100 μM VKORC1L1-siRNA in culture medium was applied for transfection (Qiagen predesigned siRNA #S104138407).

Antioxidant supplementation- Vitamin K1 and K2 concentrations were measured for neat fetal bovine serum (FBS) by extraction into isopropanol:hexane::3:2 and standard workup for HPLC-based determination as for the VKOR enzymatic assay (see following VKOR method). Mean nominal Q10 concentration in FBS was taken to be 50 nM from a previous report(14). Nominal concentrations of antioxidants in culture media supplemented to 10% FBS were: K1, 24 pM; K2, undetectable; Q10, 5 nM. For experiments requiring elevated antioxidant concentrations, antioxidants were additionally supplemented with 1 μM in culture media.

Relative quantification of mRNA by real-time RT-PCR- Total cDNA of control and hydrogen peroxide-treated (75μM) HEK 293T cells was synthesized by reverse transcription using random primers and hexanucleotides (Omniscript RT Kit, Qiagen) after isolating mRNA from cells using the RNeasy Mini Kit (Qiagen). Transcription rates of VKORC1, VKORC1L1, and the porphobilinogen deaminase (PBGD) gene, an endogenous, constitutively transcribed control, were analyzed by hydrolysis probe-based real-time PCR (ABSOLUTE™ QPCR, Abgene; see online Supplement for primers, probes and temperature cycling details). Real-time detection of amplification was performed in triplicate on an ABI Prism 7500 Sequence Detection System (Applied Biosystems). VKORC1 and VKORC1L1 transcription rates were calculated by the comparative Ct (ΔΔCt) method and normalized to PBGD expression levels relative to the zero-time (t=0) control samples(15).

VKOR and VKR activity assays for HEK 293T cell crude membranes- VKOR activity of crude cell membranes was measured by the standard DTT-driven method(16). Epoxidation of vitamin K1 and K2 (Sigma-Aldrich) was performed according to Tishler et al. to yield the respective vitamin K 2,3-epoxide (K>O)(17). Vitamin K reductase (VKR) activity was determined by the same method as for VKOR activity, but with vitamin K quinone replacing K O as substrate. Enzymatic activities to reduce K vitamin epoxides to the respective quinones, or to reduce K1 quinone to K1 hydroquinone, were driven by addition of 5mM DTT, 1 hr,
30°C as previously described(16). After organic phase extraction with isopropanol:hexane::3:2 and dry-down in vacuo, products were dissolved in methanol and resolved by C_{18}RP-HPLC chromatography (Supelcoil LC-18-S column, 150 mm x 4.6 mm, 5 μm), isocratic methanol elution, quantitated by diode array detection at 254 nm (Hitachi LaChrom Elite HPLC system, VWR, Darmstadt, Germany). K vitamins and epoxides were resolved by RP-HPLC (LaChrom Elite®, VWR-Hitachi, Darmstadt, Germany), quantification by flow-cell spectrophotometry (L-2455 diode array detector) integration of peak area A_{288nm} (t_{elution} ~10 min). Vitamin K hydroquinone was detected by flow-cell fluorometry (L-2485 fluorescence detector; λ_{ex} 240 nm, λ_{em} 430 nm, t_{elution} ~2.4 min).

Protein concentrations were determined by standard Lowry protein assay to normalize activities with respect to total protein concentration(18). VKOR kinetics for VKORC1L1 were determined for various concentrations of K vitamin epoxides (0μM - 16μM); pseudo first-order reaction conditions (i.e., quinone product concentration negligible compared to epoxide substrate concentration) at maximal velocities were verified by Eisenthal & Comish-Bowden direct linear plots. The apparent kinetic constants K_{m} and V_{max} were calculated from the intersection loci of linear regression line fits to the raw data (velocity versus [substrate]): x-intercept, mean measured velocity (nmol/mg enzyme/hr); negative y-intercept, mean substrate concentration (μM). Error ranges are ±s.e.m. for the range of data at each intersection locus.

Cell viability analysis- Cell viability was measured using the CellTiter 96® AQueous cell proliferation assay (Promega, Mannheim, Germany) according to the manufacturer’s instructions. HEK 293T cells overexpressing VKORC1L1, anti-VKORC1L1 siRNA (HP GenomeWide siRNA, Qiagen, Hilden, Germany), or random sequence siRNA (negative control) were cultured 24 hr. Subsequently, cells were plated into 96 well plates (2 x 10^4 cells/well), cultured 4 hours in presence or absence of antioxidants (K_{i}, K_{2}, Q_{10} dissolved in DMSO; neat solvent without antioxidants used as control), 37 °C in MEM, and subsequently exposed to 0 or 25 μM hydrogen peroxide for 18 hr. Viability determination began with addition of 15 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reagent, incubated at 37°C, 2 hours, followed by microplate reader absorbance measurement (490 nm) (MRX, Dynatech Laboratories, Denkendorf, Germany) to detect the colored formazan product produced by endogenous dehydrogenases in metabolically active cells.

Reactive oxygen species quantitation- Intracellular ROS was quantitated by fluorometric detection of intracellular H_{2}DCF-DA dye (Invitrogen, Karlsruhe, Germany)(19,20). Accordingly, 2 x 10^4 cells per well were pre-incubated 3 hours in MEM supplemented with 1μM K_{i}, K_{2} (MK-4), Q_{10} (Sigma-Aldrich) or with neat solvent ([DMSO]final <0.1% v/v). Cells were loaded 30 min, 37 °C with H_{2}DCF-DA, 8 μM final concentration in HBSS, washed 3 times with HBSS before addition of the aqueous-soluble free radical generator 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, Merck, Nottingham, UK) in culture medium without FBS. ROS formation was detected by conversion of 2',7’-dichlorodihydrofluorescein to fluorescent dichlorofluorescein (λ_{ex} = 480 nm, λ_{em} = 510 nm)(19), 37°C using a microplate fluorescence reader (Fluoroskan Ascent FL, Thermo Fisher GmbH, Dreieich, Germany)(20,21).

Intracellular protein carbonylation quantitation- Protein carbonyl moieties were measured in crude membranes of HEK 293T...
cells, with or without VKORC1L1 overexpression or knock-down and/or 1 µM supplemented K$_i$, using the BioCell Protein Carbonyl Assay Kit (Cell Biolabs, Inc., San Diego) according to the manufacturer's instructions for low protein level and fluorescence plate reader (Fluoscan Acent FL, Thermo Electron Corp., Thermo Fisher Scientific Germany Ltd. & Co. KG, Bonn, Germany). Dinitrophenylhydrazine (DNP) derivatized carbonyl groups were detected by a fluorescent DNP-specific antibody-based ELISA, $\lambda_{ex}$ 485 nm, $\lambda_{em}$ 538 nm (Gentaur, Brussels, Belgium). Forty-eight hours post-transfection with VKORC1L1, anti-VKORC1L1 siRNA, or empty plasmid, cells were washed twice with 0 ºC PBS, harvested, mechanically lysed in 25mM imidazole (pH 7.5), 0.5% CHAPS.

Prediction of VKORC1L1 secondary structure and topology- VKORC1L1 was recently reported to be exclusively localized to ER membranes in HeLa cells(22). We confirmed similar results for subcellular localization of VKORC1L1 in PtK2 cells (Fig. S1). Because of high sequence homology to VKORC1, for which we reported topology and secondary structure predictions in a recent study(23), we were able to predict secondary structural elements and a topological model for VKORC1L1 (Fig. 1) by alignment of VKORC1 and VKORC1L1 primary sequences to the proVKOR sequence (Fig. S2), for which a high-resolution structural model was published(8).

Bioinformatics and data analysis- Human and mouse tissues and cell-specific mRNA expression profiles for VKORC1L1 and VKORC1 were obtained from the BioGPS web server whole-genome gene expression array results (44,775 human and 36,182 mouse transcripts, 79 human and 69 mouse tissues/cell types) at http://biogps.gnf.org/. Genomics Institute of the Novartis Research Foundation(24). Multiple sequence alignment was performed using Jalview 2.6.1 (http://www.jalview.org/)(25). Statistical analyses were performed using KaleidaGraph 4.04 (Synergy Software, Reading, Pennsylvania, USA). Standard error of the mean (s.e.m.) was calculated for all replicate data and is indicated with error bars for all figures except where specifically noted in figure legends. Unpaired Student's t-test (for time-course data) and ANOVA with post hoc Fisher's Least Significant Difference test (for matched data groups) were used to assess statistical significance between data mean values as indicated by calculated p-values.

RESULTS

VKORC1L1 catalyzes both de-epoxidation of vitamin K-epoxide and reduction of vitamin K quinone. To directly assess VKORC1L1 function, we began by exploring if VKORC1L1 could support VKOR and vitamin K quinone reductase (VKR) enzymatic activities similar to those catalyzed by VKORC1 and known to be critical for haemostasis(11,26). Table 1 summarizes our experimentally determined effective Michaelis-Menten enzyme kinetics for dithiothreitol (DTT) driven VKOR activity under pseudo first-order conditions and maximal initial velocity for VKORC1L1 overexpressed in HEK 293T cells, but where concentration of the K quinone product does not appreciably compete for enzyme as a result of the alternative VKR reaction catalyzed by the enzyme. Under these conditions we found VKORC1L1 to have 2.2- and 7.3-fold lower affinity for K$_i$>O and K$_s$>O substrates, respectively, than VKORC1, suggesting that VKORC1 has evolved greater efficiency for VKOR enzymatic activity than VKORC1L1. We also measured warfarin inhibition (5 µM inhibitor in culture medium) for VKORC1L1 that was 1.8-fold lower than that for the equivalent amount of VKORC1 overexpressed in the same HEK 293T cell line (Table 1). Additionally, we semiquantitatively confirmed VKR activity for VKORC1L1 (Fig. 2).

However, we recently reported that VKORC1L1 expression in vivo cannot support sufficient physiological VKOR activity in VKOR$^{-}$ knockout mice to rescue a dyscoagulation phenotype that results in early post-partum death(27). Thus,
VKOR enzymatic activity does not appear to be the primary physiological function of VKORC1L1.

Oxidative stress induces VKORC1L1 expression. We measured in vitro VKOR activity to confirm functional expression of VKORC1L1 in HEK 293T cells treated with 75 µM H2O2 that induces intracellular oxidative stress and found VKOR activity increased nearly 5.5-fold (p<0.0001) by 120 min (Fig. 3a). In order to determine if VKORC1L1 or VKORC1 contributed to the elevated VKOR activity, we measured VKORC1 transcript levels upon H2O2 treatment by quantitative PCR of split samples used for the VKOR activity measurements and found that VKORC1 expression was actually down-regulated by 70% (p<0.0001) by 60 min before returning to basal levels from 90 through 120 min (Fig. 3b), suggesting that increased VKOR activity was due to increased VKORC1L1 expression. Direct measurement of VKORC1L1 transcript levels confirmed a 5.2-fold (p<0.0005) increase by 40 min after inducing oxidative stress with H2O2 (Fig. 3c). Taken together, these data confirm that upregulated VKORC1L1 expression was responsible for the rise in intracellular VKOR activity induced by H2O2 treatment.

VKORC1L1-mediated intracellular antioxidative effects are vitamin K-dependent. We next established the contributions of lipidic quinone antioxidants and VKORC1L1 expression level, alone and in concert, to cell viability in the absence or presence of H2O2-induced ROS stress. In the absence of applied H2O2, HEK 293T cell viability was affected by the supplemented antioxidants according to the increasing rank order: control ≈ Q10 < K1 (115% of control, p<0.05) < K2 (122% of control, p<0.05) (cf. Fig. 3, four left-most grey bars), suggesting that K1 and K2, but not Q10, could alleviate experimentally elevated levels of intracellular oxidative stress, leading to increased viability. Upon H2O2 treatment, cell viability was affected in the increasing rank order: control (69% of the untreated value, p<0.0005) < K1 (77% of the untreated K1-supplemented value, p<0.0001) < K2 (~100%, i.e., not significantly different from the untreated K2-supplemented value) < Q10 (~100%, i.e., not significantly different from the untreated Q10-supplemented value) (cf. Fig. 3, four left-most grey/white pairs of bars). Taken together, the results show that lipidic quinones supplemented at supraphysiological concentration of 1 µM in cell culture medium affect cell viability in the presence of physiological or H2O2-induced, elevated intracellular ROS levels in identical rank order: K2 > K1 > Q10. The positive effect of quinone antioxidants on cell viability is, at least in part, VKORC1L1-dependent. Although viability of unstressed cells that over-express VKORC1L1 was not statistically different than that for untreated control cells (Fig. 3d, cf. control and L1 grey bars), H2O2-treatment did not reduce viability for cells that over-express VKORC1L1 (Fig. 3d, cf. grey and open L1 bars), suggesting that VKORC1L1 mediates vitamin K-dependent cellular homeostasis mechanisms during oxidative stress, and that trace amounts of bioavailable K1 in FBS-supplemented culture medium (24 pM measured, see Materials & Methods) are not limiting to the VKORC1L1-mediated antioxidant effect. Contrastingly, although viability of VKORC1L1 knock-down cells in both presence and absence of 1 µM supplemented K1 was not significantly different from the value for untreated control cells (Fig. 3d, cf. control, siL1 and siL1+K1 grey bars), there was a small, but significant, measurable difference in the viability for H2O2-stressed VKORC1L1 knock-down cells (Fig. 3d, 84% of untreated siL1 viability, p<0.05; cf. grey/white pair of bars for siL1), and for H2O2-stressed VKORC1L1 knock-down cells supplemented with K1 (Fig. 3d, 84% of untreated siL1+K1 viability, p<0.05; cf. grey/white pair of bars for siL1+K1), compared to the respective unstressed controls. Taken together, these results suggest that VKORC1L1 knock-down does not have a negative impact on cell viability during unstressed growth, although VKORC1L1 is apparently required for the vitamin K-mediated effect on cell viability
because 1 μM supplemented K₁ had no effect on the siL₁+K₁ cells. Also, upon H₂O₂-treatment, 1 μM supplemented K₁ did not significantly increase viability relative to VKORC1L1 knock-down cells without supplemented K₁ (Fig. 3d, cf. right-most two white bars), suggesting that VKORC1L1 is required for vitamin K-dependent increased cell proliferation.

**Increased VKORC1L1 expression level correlates with reduced intracellular ROS level.** We further investigated the effects of the various lipidic small-molecule antioxidants and VKORC1L1 expression level on increased intracellular ROS level induced by cell uptake and redox cycling of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). Intracellular ROS level was assessed by fluorescence report of oxidation of intracellular 2',7'-dichlorodihydrofluorescein to 2',7'-dichlorofluorescein by free radicals. In the presence of nominal antioxidant concentrations in the culture medium, and relative to wild-type control cells, ROS levels were reduced by 23% (p<0.0001) or elevated by 9% (p<0.0001) in cells overexpressing VKORC1L1 or transfected with anti-VKORC1L1 siRNA, respectively (Fig. 3e, left-most group "Untreated", white and grey bars). We found that protein overexpression, as such, was not responsible for elevated ROS levels when we overexpressed γ-glutamyl carboxylase (GGCX), a vitamin K cycle enzyme that can oxidize vitamin K hydroquinone, as a control (stippled bar). Taken together, these results indicate that the ROS-scavenging antioxidative effect of endogenous K₁ and/or Q₁₀ (both nominally supplied from culture medium, Q₁₀ also endogenously synthesized in cells) are potentiated in a dose-dependent manner by VKORC1L1. Supplementation of 1 μM K₁, K₂, and Q₁₀ to DMNQ-treated cells resulted in lowered ROS levels by 18% (p<0.0005), 33% (p<0.0001), and 12% (p<0.05), respectively, compared to the untreated wild-type control (Fig. 3e, black bars). Thus, for HEK 293T cells expressing constitutive levels of protein, the rank order for effectiveness of lipidic antioxidants in lowering DMNQ-reported free radicals is K₂ > K₁ > Q₁₀.

Additionally, combined effects of VKORC1L1 protein overexpression or knock-down together with supplementation of individual lipidic antioxidants (Fig. 3e, compare triplets of black, open, grey bars for each antioxidant) revealed altered ROS levels of -16% (L₁, p<0.001) and +20% (siL₁, p<0.001) for K₁-treated cells, +58% (siL₁, p<0.0001) for K₂-treated cells (L₁ data did not reach statistical significance), and -19% (L₁, p<0.001) for Q₁₀-treated cells (siL₁ data did not reach statistical significance). These results strongly suggest that the antioxidant effects are dependent on VKORC1L1 expression level. Thus, where differences in measured levels of DMNQ-reported ROS were found to be statistically significant between constitutively expressing cells and VKORC1L1 overexpressing or knock-down cells, the same trends in VKORC1L1 overexpression resulting in lowered ROS levels, as well as in VKORC1L1 knock-down resulting in increased ROS levels, were observed for each of the three supplemented lipidic antioxidants. These results, taken together with localization of VKORC1L1 and lipidic quinones together in ER membranes, strongly suggests that VKORC1L1 plays a fundamental role in mediating antioxidative effects in cellular membranes.

Interestingly, although Q₁₀ supplementation alone had no significant effect on cell viability relative to untreated controls (see Fig. 3d and results from the previous section), VKORC1L1 overexpression together with applied 1 μM Q₁₀ resulted in a considerably lowered ROS level (-19%, p<0.001) relative to the Q₁₀-treated wild-type control (Fig. 3e, cf. right-most black and open bars). However, Q₁₀ has been reported to be a substrate for a homologous prokaryotic VKOR enzyme(8). Thus, the antioxidant effect of Q₁₀ revealed in this study might be synergistic with VKORC1L1-mediated reduction of vitamin K, or Q₁₀ might be a physiological substrate for VKORC1L1 in addition to K₁ and K₂. Our study does not discriminate between these two possibilities.
Taken together, the results presented in Figure 3e indicate that the VKORC1L1-dependent antioxidative effect, reflected in ROS level as a biomarker, scales with VKORC1L1 expression level and is mediated by K vitamins, and possibly also by Q10, over the experimentally tested ranges for both factors. VKORC1L1 expression level inversely correlates with oxidative protein damage. In order to directly assess the role of VKORC1L1 in alleviating intracellular ROS-mediated damage, we directly measured membrane protein carbonylation (Fig. 3f), which results from free radical attack on unsaturated bonds in proteins. Overexpression of VKORC1L1 alone resulted in 40% (p<0.01) reduction in carbonylation level, while overexpression of VKORC1L1 together with 1 µM K1 resulted in 71% (p<0.0001) reduced carbonylation, compared to untreated control cells in the presence of endogenous K1 in normal culture medium. Knock-down of VKORC1L1 resulted in a marginal (10%) increase of mean measured protein carbonylation relative to untreated controls, but the level of statistical significance for the data does not provide confidence on this point.

DISCUSSION

Previous work by Li et al. demonstrated that cell death caused by oxidative stress can be prevented by low nanomolar concentrations of vitamins K1 and K2 in cultured neurons and oligodendrocytes(4). Furthermore, seminal studies by Mukai et al. reported KH2 to have a greater capacity than QH2 to regenerate α-tocopherol (αT) from the α-tocopheryl radical (αT•) resulting from the major physiological free radical scavenging pathway(28,29). Therefore, we hypothesized that the specific function of VKORC1L1 is to keep the intracellular intramembranous pool of K vitamins in the reduced, antioxidant-active hydroquinone form.

In this study we have studied cells exposed to sustained, supraphysiological ROS (H2O2, DMNQ) levels in order to reveal a direct role for VKORC1L1 in mediating intracellular antioxidative effects. Contrastingly, there have been numerous reports confirming the roles of transient, low ROS levels in cell signaling pathways involving complex regulation of transcription factor networks(30,31). Most recently, studies have shown ROS signaling to be important to cell survival and proliferation(32,33). We recognize that, in addition to the VKORC1L1-mediated antioxidative function we revealed, various signaling pathways sensitive to the lipidic quinone antioxidants used in our study may also play a role in the effects on cell viability. For example, the steroid and xenobiotic SXR receptor, upon binding vitamin K2, was found to upregulate CYP3A4 expression and regulate transcription factor expression involved in bone homeostasis(34-36). However, the data we present here on actual intracellular ROS level and oxidative protein damage, complimented by the cell viability data, are all consistent with a direct role for VKORC1L1 in antioxidation.

Evidence that VKORC1L1 functions to maintain vitamin K in the reduced, active antioxidant state. For wild-type cells we found that oxidative stress induced by H2O2 dramatically upregulated VKORC1L1 expression (Fig. 3c) accompanied by increased VKOR enzymatic activity (Fig. 3a). We also found that VKORC1L1 supports VKR activity (Fig. 2), implying that VKORC1L1-dependent VKR activity also increases upon H2O2 exposure. Taken together, these results suggest that oxidative stress increases VKORC1L1 expression which can increase intracellular levels of reduced vitamin K cofactors which, in turn, directly or indirectly lower intracellular ROS levels (Fig. 3e) and ROS-induced protein damage (Fig. 3f). Additionally, we determined that increased VKORC1L1 expression mediates increased cell viability that is vitamin K-dependent under conditions of experimentally induced oxidative stress (Fig. 3d) that strongly suggests a regulatory role for VKORC1L1 in intracellular redox homeostasis.
In support of a fundamental homeostatic role for VKORC1L1, results from a previous high throughput human genome-wide expression study indicate that VKORC1L1 is expressed at uniform levels in nearly all tissues and cell types, while VKORC1 exhibits greater tissue-specific variation in expression level (Fig. S3)(24). Ubiquitous, constitutive expression of VKORC1L1 in nearly all investigated human tissues likely points to an important metabolic house-keeping function (Fig. S3a). Contrastly, there were significantly high VKORC1L1 expression levels (Fig. S3a, light green bar >3-fold above mean) in highly redox-active brown fat adipocytes, and significantly elevated levels in monocytic CD34+ cell lines and B lymphoblasts (Fig. S3a, blue bars). Surprisingly, these results correlate well with recent reports of intense and protracted ROS bursts that are characteristic of these cell types during normal physiological function(37-40). Thus, independent results from these studies further point to specific roles for VKORC1L1 in intracellular redox homeostasis as well as in response to acute ROS-induced oxidative stress.

We found further *in vivo* evidence for VKORC1L1 playing a central role in intracellular antioxidation in previous study data of transcription levels in mouse mast cells. Mast cells have recently been shown to generate prolonged, high concentrations of ROS upon activation by binding IgE on the cell surface(39,40). Several years earlier, the results of a genome-wide high-density oligonucleotide array study of mouse mRNA expression included data for both VKORC1L1 and VKORC1 in mast cells at rest or activated by IgE (Fig. S4)(24). These data showing up-regulation of VKORC1L1, but not VKORC1, expression during IgE binding-induced ROS stress in mouse mast cells are in agreement with our finding that VKORC1L1 expression is upregulated during oxidative stress, and further supports our conclusion that VKORC1L1 plays a fundamental role in intracellular antioxidation. Therefore, VKORC1L1 upregulation during oxidative stress could effectively increase the fraction of KH₂ in the intracellular vitamin K pool that would serve to protect membrane lipids and proteins from oxidative damage either directly, or indirectly through regenerative cycling of lipid membrane-resident ubihydroquinone (Q₁₀H₂), E vitamins and ascorbate antioxidant species (Fig. S5). In fact, K vitamins have recently been shown to directly regenerate reduced ubihydroquinone (Q₁₀H₂) and α-tocopherol (αT) in both polar and nonpolar environments, the later at a significantly faster rate (8x10⁶ M⁻¹s⁻¹) than the well characterized αT/ascorbate regenerative system (3x10⁶ M⁻¹s⁻¹; see Fig. S5 comparing rates of free radical scavengers)(28,29). This suggests that overexpressed VKORC1L1 might indirectly mediate reduction of Q₁₀ by directly reducing endogenous K₁ which, in turn, could keep the intracellular Q₁₀ pool reduced.

*Various physiological partner oxidoreductases provide reducing equivalents to VKOR enzymes.* Although all prokaryotic and eukaryotic VKOR primary protein sequences share highly similar predicted structural elements with their VKOR core domains comprising a four transmembrane (TM) α-helical bundle, two conserved cysteines and a conserved serine/threonine in the large 40-50 residue loop connecting the first two TM helices, and two additional conserved cysteines in a CXXC reaction center located in the fourth TM helix (*cf.* Fig. 1, Fig. S2), distinct functional differences have already been reported between proVKOR from VKORC1.

For example, VKORC1 has been shown to obtain reducing equivalents from the ER lumen from protein disulfide isomerase (PDI), the major ER-resident oxidoreductase responsible for OPF in rat hepatocytes(41), and transfers these to K₁ or to K in the ER membrane(42). Very recently, membrane-intrinsic thioredoxin-like TMX4, TMX4 and ERIp18 oxidoreductases were shown to be preferred physiological redox partners for human VKORC1 that was heterologously expressed in monkey-derived COS-7 cells(43). In fact, VKORC1 and VKORC1L1 might functionally interact with as many as 19 PDI-family proteins in human
cells(44). In contrast, in vivo proVKOR obtains reducing equivalents from its C-terminal thioredoxin-like domain or from separate, soluble periplasmic oxidoreductases such as DsbA to reduce ubiquinol (QH₂) in the bacterial periplasmic membrane(45). However, as K=O is not found in any known prokaryotic lipidome(46) and functional enzymes responsible for γ-glutamyl carboxylation of proteins are absent from prokaryotic genomes(47), proVKOR function must be distinctly different from that of VKORC1.

Substrate specificity varies among VKOR enzymes. Heterologously expressed proVKOR from E. coli was reported to pass reducing equivalents in vitro to vitamins K₁ and K₂ in addition to Q₁₀, but not to K=O(8). Therefore, proVKOR apparently does not possess VKOR (de-epoxidase) activity and should technically not be called proVKOR, but rather proVKR, as it catalyzes only quinone to hydroquinone reduction. Ultimately, both VKORC1 and proVKOR enzymatic activities result in transfer of reducing equivalents from cysteines incorporated into protein disulfides to quinone cofactors in the ER membrane. Thus, quinone cofactor usage in VKORC1 appears to have been neofunctionalized or subfunctionalized since its divergence from the last universal common ancestor (LUCA) common to itself and proVKOR.

A proposed Grande Vitamin K Cycle in vertebrates. In the presence of intracellular dissolved O₂, GGCX incorporates dissolved CO₂ into γ-glutamyl carboxylate groups of post-translationally modified vitamin K-dependent proteins, consuming KH₂ and O₂ to produce K=O and water as by-products. KH₂ is also likely consumed in the ER membrane through its antioxidative action and ultimately results in conversion to oxidized K, likely via collisional dismutation or possibly nonenzymatic reduction of intermediate K• radicals. VKOR paralog enzymes are coexpressed in all tissues, but at relatively uniform levels for VKORC1L1, while at widely varied tissue-specific levels for VKORC1 (cf. Figs. S3a, S3b). Both enzymes possess both VKOR and VKR activities, although VKORC1 appears to be specialized to perform the VKOR reaction at high turnover rates relative to VKR activity(26) (Fig. 5), while we propose that VKORC1L1 is specialized to carry out the VKR reaction at high turnover rates relative to VKORC1L1-specific VKOR activity measured in the present study. Consistent with this view, we measured higher apparent Kₘ and Vₘₐₓ values for VKOR activity of VKORC1L1 compared to values measured for VKORC1 (Table 1). Additionally supporting this scheme are the turnover rates we calculated from previous results of Chu et al. for measured VKR (46.4•10⁻³ mol⁻¹VKORC1•hr⁻¹) and VKR (85.7•10⁻⁶ mol⁻¹VKORC1•hr⁻¹) enzymatic activities of purified, reconstituted VKORC1, indicating that VKR activity represents a turnover rate ~2.5 orders of magnitude (541-fold) slower than that for VKOR activity(26). Surprisingly, we measured a relatively low VKOR activity for VKORC1L1 with a turnover of 2.57•10⁻⁶ mol⁻¹VKORC1•hr⁻¹, equivalent to >4 orders of magnitude (18,054-fold) slower turnover than for that of VKORC1. To explain this low VKR activity, and as VKORC1L1 was reported not to be able to support adequate VKOR activity to substitute for the function of VKORC1 in VKORC1⁻/⁻ mice(27), we hypothesize that VKR enzymatic activity is the primary physiological function of VKORC1L1. This hypothesis is consistent with our findings that VKORC1L1 supports vitamin K-mediated intracellular antioxidative mechanisms. In summary, Figure 5 illustrates our current working hypothesis of how both VKOR enzyme paralogs catalyze the same pair of reactions, yet possess complimentary functions to maintain the intracellular vitamin K pool in the fully reduced hydroquinone form. Accordingly, VKORC1 is more efficient at de-epoxidation (i.e., VKOR activity) while VKORC1L1 is more efficient at quinone to hydroquinone reduction (i.e., VKR activity).

In conclusion, VKORC1L1 appears to be responsible for transfer of reducing equivalents, generated as by-products of OPF, to vitamin K-mediated intracellular antioxidation pathways. The involvement of
VKORC1L1 and K vitamins in antioxidation pathways warrants closer investigations to answer still open questions about relative amounts and dynamics of small molecule antioxidants in various subcellular compartments. Similarly, localization, expression levels and regulatory control of other enzymes involved in maintaining lipidic quinones in the respective reduced, active antioxidant forms deserves closer scrutiny. Moreover, the specific impact of VKORC1L1 and physiological levels of K vitamins on cellular damage inflicted by reactive oxygen species, and the general impact on aging and age-related chronic diseases including cancer, cardiovascular disease and neurodegeneration requires future detailed assessment. Finally, the discovery of the antioxidant-regenerative role of VKORC1L1 will require a major reappraisal of the fundamental concepts of intracellular redox homeostasis.
REFERENCES


FOOTNOTES

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‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed:

† The abbreviations used are: DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; K1, vitamin K1=phylloquinone; K2, vitamin K2=menaquinone; KH2, vitamin K hydroquinone; OPF, oxidative protein folding; proVKOR,
prokaryotic VKOR enzyme; $Q_{10}$, ubiquinone-10; $Q_{10}H_2$, ubihydroquinone-10; ROS, reactive oxygen species; siRNA, small interfering RNA; VKOR, vitamin K 2,3-epoxide reductase; VKORC1, vitamin K 2,3-epoxide reductase complex subunit 1; VKORC1L1, vitamin K 2,3-epoxide reductase complex subunit 1-like 1; VKR, vitamin K quinone reductase.

¶ All numerical percentage (%) values appearing in the Results section refer to comparisons between mean data values for the respective groups where statistical significance is reported as a calculated p-value in each case.
FIGURE LEGENDS

Fig. 1. A topological model for human VKORC1L1 based on sequence alignment (Fig. S2) to a prokaryotic VKOR homolog protein structure determined to 3.6 Å resolution. Circles represent amino acid residues; bold circles indicate sequence identity shared by VKORC1L1 and paralog VKORC1; TM1-TM4, first through fourth transmembrane α-helices; black filled circles, residues completely conserved among all VKOR family proteins; grey boxed region, the catalytic CIIC motif; black boxed regions, putative warfarin binding site.

Fig. 2. VKORC1L1-mediated reduction of K1 quinone to K1 hydroquinone in HEK 293T whole cell membranes. C18RP-HPLC chromatograms (overlaid) showing the fluorescence peak of K1H2 at ~2.4 min for wild-type HEK 293T cell membranes incubated with DTT in the absence of K1 substrate (blue trace), with DTT and K1 (black trace), and for HEK 293T cells over-expressing VKORC1L1 incubated with DTT and K1 (red trace).

Fig. 3. Experimentally measured time-dependent VKOR activity, VKORC1L1 and VKORC1 mRNA levels, cell viability, intracellular ROS levels and membrane protein carbonylation from lysates of HEK 293T cells overexpressing VKORC1L1 with or without antioxidant supplementation. a, DTT-driven VKOR activity (n number of replicate measurements; n=2 for each time point) for crude membranes of VKORC1L1-expressing cells prepared 0 min through 120 min post-treatment with 75 μM H2O2. b, Quantitative PCR data (n=3 for each time point) for VKORC1 mRNA expression from aliquots of same cells assayed for VKOR activity in (a), 0 min through 120 min post-treatment with 75 μM H2O2. c, Quantitative PCR data (n=3 for each time point) for VKORC1L1 mRNA expression from aliquots of same cells assayed for VKOR activity in (a), 0 min through 90 min post-treatment with 75 μM H2O2. d, Cell viability assay data (n=21 for Control, K1, L1, siL1, siL1+K1; n=5 for K2, Q10) for HEK 293T cells exposed to biological quinones or with exogenously overexpressed or silenced VKORC1L1 in the presence or absence of 25 μM H2O2. Control, untreated cells; K1, cells with 1μM phylloquinone in the medium; K2, cells with 1μM menaquinone-4 in the medium; Q10, cells with 1μM ubiquinone-10 (coenzyme Q10) in the medium; L1, cells overexpressing VKORC1L1; siRNA L1, cells expressing silencing RNA for VKORC1L1; siRNA L1 +K1, cells expressing silencing RNA for VKORC1L1 with 1μM phylloquinone in the medium; grey-filled bars, culture medium without H2O2; open bars, culture medium with 25 μM H2O2. e, Influence of VKORC1L1 expression and applied antioxidants on ROS generation induced by 100 μM DMNQ in HEK 293T cells measured as dichlorohydrofluorescein fluorescence (λex 488, λem 525 ). Bars indicate mean values (n=8 for all samples except siL1+Q10 for which n=7) of intracellular ROS measured by H2DCF-DA fluorescence for cells with the following attributes: black bars, wild-type cells; open bars, cells overexpressing VKORC1L1; grey bars, cells expressing anti-VKORC1L1 siRNA; bar with horizontal hatching, cells overexpressing γ-carboxylase (GGCX). Groups of bars represent the following antioxidants added to culture media: control, without added antioxidant; K1, with 1 μM added phylloquinone; K2, with 1 μM added menaquinone-4 ; Q10, with 1 μM added ubiquinone-10 (coenzyme Q10). f, Influence of VKORC1L1 overexpression and antioxidants on protein carbonylation. Bar heights represent mean DNP-derivative carbonyl groups measured by anti-DNP ELISA assay (n=4) for HEK 293T cells with the following culture conditions: control, wild-type cells with no added antioxidants; control +K1, wild-type cells with 1 μM added phylloquinone; L1, cells overexpressing VKORC1L1; L1 +K1, cells overexpressing VKORC1L1 with 1 μM added phylloquinone; siL1, cells expressing siRNA against VKORC1L1; siL1 +K1, cells expressing siRNA against VKORC1L1 with 1 μM added phylloquinone. All measurements reported as arbitrary units were normalized to the initial mean control values. Error bars, ± s.e.m.
Fig. 4. Enzymatic pathways involving oxidative protein folding and K vitamins in the ER. ER membrane bilayer represented as grey bar with embedded proteins (open, white shapes) and K vitamin species (black hexagons). Soluble ROS molecules (O$_2^•$- and H$_2$O$_2$) and proteins are indicated in the ER lumen below the ER membrane. PDI, protein disulfide isomerase (conflicting reports suggest that PDI in microsomes from rat liver cells (41) or human TMX, TMX4, and ERp18 heterologously expressed in monkey kidney-derived COS-7 cells (43) are the physiological redox partner proteins for rat VKORC1 and human VKORC1, respectively); proteins that can be oxidatively folded incorporating disulfide bridges are indicated as black curves (reduced forms) and spirals (folded, oxidized forms). Arrows indicate directional flow of reducing equivalents between enzymes and K vitamin species, and transitions between reduced and oxidized states for PDI (ox, oxidized; red, reduced). The exchanged reducing equivalent species are indicated in parentheses. Enzymes (left to right): QSOX1a,2, quinone:sulfide oxidoreductase paralogs 1a and 2 (both bitopic membrane proteins anchored in the membrane by a single transmembrane α-helix; NQO1, NAD(P)H:quinone oxidoreductase 1 (formerly DT-diaphorase); CYP1A1, cytochrome P450 oxidase isoform 1A1; PDI, protein disulfide isomerase 1A (equivalent to proline 4-hydroxylase β subunit); VKORC1, vitamin K 2,3-epoxide reductase complex subunit 1; VKORC1L1, vitamin K 2,3-epoxide reductase complex subunit 1 like-1; ERO1Lα,β, endoplasmic oxidoreductin-1 paralogs α and β. Gla stands for γ-carboxyglutamyl residues of vitamin K-dependent post-translationally modified proteins; Glu stands for unmodified target glutamic acid residues of vitamin K-dependent post-translationally modified proteins. The PDI redox cycle is explicitly depicted only for EroL1α,β and VKORC1L1; VKORC1 interacts similarly with PDI where only the oxidized and reduced forms are shown for economy of space in the figure. The classical vitamin K cycle heretofore described in the literature involving diffusion of K vitamers between VKORC1 and GGCX is shown at the right.

Fig. 5. A proposed Grande Vitamin K Cycle. Proposed functional relationships between vertebrate VKORC1L1 and VKORC1 paralog enzymes, GGCX responsible for post-translational protein γ-glutamyl carboxylation, and reducing equivalent exchange functions of K vitamins in the ER membrane: Glu, glutamyl protein residue; Gla, γ-carboxylated glutamyl protein residue; K, vitamin K quinone; KH$_2$, vitamin K hydroquinone; K>0, vitamin K 2,3-epoxide. Enzymes depicted as ovals: GGCX, γ-glutamyl carboxylase; VKORC1, vitamin K 2,3-epoxide reductase complex subunit 1 (red ovals represent enzymes of the "classical" vitamin K cycle); VKORC1L1, VKORC1-like 1 (green oval). Thick arrows indicates relative turnover rates of naphthoquinone substrates K>0 and K by VKORC1L1 (lower pair of arched arrows) and VKORC1 (upper pair of arched arrows). Antioxidative function of KH$_2$ depicted as green sunburst.
### Table 1. VKOR *in vitro* reaction enzymatic parameters for human enzymes overexpressed in HEK 293T cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate†</th>
<th>Inhibitor</th>
<th>$K_m^*$ (µM)</th>
<th>$V_{max}^*$ (nmol•mg⁻¹•hr⁻¹)</th>
<th>% Inhibition‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>VKORC1L&lt;sub&gt;1&lt;/sub&gt;</td>
<td>K1&gt;O</td>
<td>-</td>
<td>4.15 ± 0.10</td>
<td>2.57 ± 0.05</td>
<td>0</td>
</tr>
<tr>
<td>VKORC1L&lt;sub&gt;1&lt;/sub&gt;</td>
<td>K1&gt;O 50 µM</td>
<td>5 µM warfarin</td>
<td>-</td>
<td>-</td>
<td>29.2 ± 7.9</td>
</tr>
<tr>
<td>VKORC1L&lt;sub&gt;1&lt;/sub&gt;</td>
<td>K2&gt;O</td>
<td>-</td>
<td>11.24 ± 0.23</td>
<td>13.46 ± 0.22</td>
<td>0</td>
</tr>
<tr>
<td>VKORC1&lt;sub&gt;1&lt;/sub&gt;</td>
<td>K1&gt;O 50 µM</td>
<td>5 µM warfarin</td>
<td>-</td>
<td>-</td>
<td>52.9 ± 6.6</td>
</tr>
<tr>
<td>VKORC1&lt;sub&gt;1&lt;/sub&gt;</td>
<td>K2&gt;O</td>
<td>-</td>
<td>1.55 ± 0.55</td>
<td>1.72 ± 0.10</td>
<td>0</td>
</tr>
</tbody>
</table>

*Apparent $K_m$ and $V_{max}$ mean values ± graphically determined error range of data scatter from Eisenthal & Cornish-Bowden direct linear plots. †Range of K>O substrate concentrations: 1-16 µM; DTT-driven (5 µM) VKOR enzymatic reaction run under pseudo first-order conditions at maximum velocity for each substrate concentration. ‡Values for % Inhibition are means ± s.e.m.
Figure 3
Human vitamin K 2,3-epoxide reductase complex subunit 1-like 1 (VKORC1L1) mediates vitamin K-dependent intracellular antioxidant function
Philipp Westhofen, Matthias Watzka, Milka Marinova, Moritz Hass, Gregor Kirfel, Jens Mueller, Carville G. Bevans, Clemens R. Mueller and Johannes Oldenburg

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