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Overexpression of protein disulfide isomerase enhances vitamin K epoxide reductase activity

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Abstract:

Vitamin K epoxide reductase (VKOR) activity is catalyzed by the VKORC1 enzyme. It is the target of vitamin K antagonists (VKA). Numerous mutations of VKORC1 have been reported and have been suspected to confer resistance to VKA and/or affect its velocity. Nevertheless, the results between studies have been conflicting, the functional characterization of these mutations in a cell system being complex due to the interweaving of VKOR activity in the vitamin K cycle.

In this study, a new cellular approach was implemented to globally evaluate the vitamin K cycle in the HEK293 cells. This global approach was based on the vitamin K quinone/vitamin K epoxide (K/KO) balance. In the presence of VKA or when the VKORC1/VKORC1L1 were knocked out, the K/KO balance decreased significantly due to an accumulation of vitamin KO. On the contrary, when VKORC1 was overexpressed, the balance remained unchanged, demonstrating a limitation of the VKOR activity. This limitation was shown to be due to an insufficient expression of the activation partner of VKORC1, as overexpressing the protein disulfide isomerase (PDI) overcomes the limitation.

This study is the first to demonstrate a functional interaction between VKORC1 and the PDI enzyme.

Keywords: Vitamin K cycle, Protein Disulfide-Isomerases, Vitamin K Epoxide Reductase, Vitamin K antagonist, Reduction

51 **Introduction:**

52 Vitamin K is a fat-soluble vitamin and the co-substrate for of vitamin K dependent proteins
53 (VKDP) activation through γ -carboxylation (Willems et al. 2014); Shearer and Newman 2008).
54 Various decisive physiological processes are linked to vitamin K (Bandyopadhyay 2008;
55 Danziger 2008; Gong et al. 2008; Willems et al. 2014). This latter was originally found to
56 contribute to haemostasis through the activation of coagulation factors II, VII, X, IX and
57 proteins C, S and Z. Subsequently, vitamin K was shown to be involved in other important
58 physiological processes, such as the prevention of arterial calcification through Matrix Gla
59 Protein (MGP), energy homeostasis through osteocalcin and apoptosis through Gas6 activation.
60 Since vitamin K diet intake is poor, vitamin K needs to be recycled through the vitamin K cycle
61 (Dam 1942; Stafford 2005) (Figure 1). The vitamin K cycle is composed of three steps. Vitamin
62 K quinone (KH_2), is converted into vitamin K epoxide (KO) by the gamma-glutamyl
63 carboxylase enzyme (GGCX). This enzymatic activity is called vitamin K dependent
64 carboxylation (VKDC). Vitamin KO is then converted into vitamin K quinone by the vitamin
65 K epoxide reductase complex subunit 1 enzyme (VKORC1) (Li et al. 2004; Oldenburg et al.
66 2000) and the vitamin K epoxide reductase complex subunit 1-like enzyme (VKORC1L1)
67 (Hammed et al. 2013; Oldenburg et al 2015). This enzymatic activity is called vitamin K
68 epoxide reductase activity (VKOR). Finally, vitamin K quinone is regenerated in KH_2 by the
69 vitamin K reductase activity (VR) supported by VKORC1, VKORC1L1, and presumably other
70 enzymes like NAD(P)H dehydrogenase quinone 1 (NQO1) (Ingram et al. 2013; Ulrich et al.
71 1988). An exogenous intake of vitamin K into the cycle is provided by the diet (Booth 2012),
72 and its cellular uptake and metabolism by cytochromes P450 enzymes have been the object of
73 several studies (Barkhan and Shearer 1977; Shearer and Newman 2008; Edson et al. 2013;
74 Goncalvez et al, 2014).

If the vitamin K cycle depends on all its constitutive enzymes, it also depends on the catalytic activation of latter. Thus, VKORC1 and VKORC1L1 need to be reduced beforehand to be functional (Rishavy et al. 2011; Schulman et al. 2010; Wajih, et al. 2006) (Figure 1). One or several protein partners could transfer their reduction power to VKOR enzyme, *via* conserved cysteines of their luminal loop. Redox partner candidates for VKORC1 have been proposed to belong to the protein-disulfide isomerase like family (PDI-like) : the protein disulfide isomerase (PDI) (Hildebrandt and Suttie 1984; Soute et al. 1992), two endoplasmic reticulum membrane-anchored proteins (Schulman et al. 2010) (TMX1 and TMX4) and two luminal proteins (ERP18 (Schulman et al. 2010) and PDIA6 (Schaafhausen et al. 2011)) have been suggested as potential redox partners. Unfortunately, no consensus has been found on their identity.

The VKORC1 is the therapeutic target of vitamin K antagonists (VKAs) (Bodin et al. 2008; Czogalla et al., 2013; D'Andrea et al., 2005; Geisen et al., 2005; Harrington et al. 2011; Hodroge et al. 2012; Limdi et al. 2008; Rieder et al. 2005; Rost et al. 2004; Schmeits et al. 2010; Watzka et al. 2011). Since the 1950s, these oral anticoagulants are prescribed all over the world to treat and prevent thromboembolism in human beings (Franchini et al. 2016; Levy et al. 2003; Van Gorp and Schurgers 2015; Vermeer and Schurgers 2000). Warfarin remains the lead VKAs and is largely prescribed (1% of the US and the UK populations) (Pirmohamed 2006a). Due to the variability in dose response in the human population, VKAs dosage needs to be individualized to each patient and adjusted using the international Normalized ratio (INR)(« Point-of-Care International Normalized Ratio (INR) Monitoring Devices for Patients on Long-term Oral Anticoagulation Therapy », 2009)(Pirmohamed 2006b). VKAs are also used as rodenticides and represent major substances for rodent population management (Bentley 1972; Isackson and Irizarry 2020). Differences in sensitivity to anticoagulant rodenticide also exist which makes rodent population control challenging (Goulois et al. 2017; Pelz et al. 2005). Mutations or polymorphisms of VKORC1 have been described to be involved

in resistance to VKAs. In humans and rodents, more than 40 mutations that could impact the enzyme velocity or inhibitory mechanism, have been reported and characterized (Hodroge et al. 2012; Oldenburg et al. 2000). Several *in vitro* assays have been used to study VKOR activity. Historically, DTT-driven enzymatic assays based on vitamin K quantification and DTT-dependent VKORC1 activation, gave important knowledge on redox transfers (Bevans et al. 2013; Friedman and Shia 1976; Hodroge et al. 2012). However, VKORC1 activation in DTT-based assay is artificial, and other biological actors of vitamin K cycle are absent. Recently, promising cellular based assays have been conducted to approach VKOR activity in a more biological way (Fregin et al. 2013; Haque et al. 2014; Rishavy et al. 2013; Tie, Jin, Tie et al. 2013). Yet, they are all indirect evaluation of the VKOR activity by dosing gamma-carboxylation of a reporter vitamin K dependent protein. Therefore, major caution is needed when measuring VKOR activity *via* these cell-based assays due to the circular aspect of vitamin K regeneration and the lack of knowledge of the cellular limiting step(s). For that reason, the results of the characterization of VKORC1 have sometimes been contradictory between thus studies and/or techniques (Czogalla et al. 2013; Hodroge et al. 2012; Tie et al. 2011). It is therefore necessary to propose a direct cell-based assay evaluating the K quinone/KO balance to identify the limiting step(s) of the vitamin cycle and characterize effects of VKORC1 mutations on the velocity and resistance to VKAs.

Material and methods

Chemicals

Vitamin K₁ quinone and warfarin were purchased from Sigma-Aldrich (France), dimethylsulfoxide, diethyl ether, methanol, ethanol, isopropyl alcohol and orthophosphoric acid from VWR International. Solution for injection of Vitamin K₁ was obtained from TVM

(France). Vitamin K₁ epoxide was synthesized according to Palmer (1984). High-performance liquid chromatography (HPLC) grade water was prepared using a milli-Q plus system (Millipore, France) and used for preparation of HPLC eluents.

Cell lines and culture

All the cell lines used in this study were cultured under humidified 5% CO₂ atmosphere in 90% of Dulbecco's Modified Eagle Medium with 4mM L-glutamine supplemented with 10% (v/v) of heat-inactivated fetal bovine serum and 100 U/mL of Penicillin-streptomycin (Gibco, France). Double-knockout HEK293 cell line was a generous gift from D.W Stafford and J. Tie.

Vitamin K equilibrium cell-based assay

Cells were grown in 6-well plates (Gauss, France). When cells reached 60% of confluency, vitamin K₁ quinone was added. After incubation, vitamins K vitamers were extracted from cells and from medium. For extracellular vitamins K, 1mL of cell culture media was used. For intracellular vitamin K, after total cell media removal (cell monolayer was wash with phosphate-buffered saline, two times), 1mL of phosphate-buffered saline, pH 7.4 (Gibco, France), was added per well. Cells were scrapped using a cell lifter (Corning, France) and counted using an automated cell counter SYSMEX XT200™ (Sysmex, France). Liquid-liquid (solvent) extraction was performed by adding 4 mL of isopropyl alcohol/hexane (1:1) per mL of medium or cells resuspended in phosphate-buffered saline. After mixing and 10 minutes of centrifugation at 3000 x g, the hexane phase was removed and dried under nitrogen gaz. Dry residues were immediately dissolved in 100μL of methanol and analyzed by liquid chromatography-mass spectrometry. Liquid chromatography-mass spectrometry (LC-MS) in atmospheric pressure chemical ionization (APCI) (LC-APCI/MS/MS 1100 Series LC/MSD ion Trap VL, Agilent technologies, France) was used to quantify vitamin K and KO as previously

described (Hammed et al. 2012; Hodroge et al. 2013). Culture medium (without vitamin K added) after 30 hours of HEK293 cell culture contained 3.31 ± 0.09 nM of vitamin K for 10^6 cells and 4.20 ± 0.8 nM of vitamin KO for 10^6 cells. Intracellular environment (without vitamin K added) after 30 hours of HEK293 cell culture contained 22.3 ± 0.02 nM of vitamin K for 10^6 cells and 0.33 ± 1.04 nM of vitamin KO for 10^6 cells. After 30 hours of incubation at 37°C in culture plate, the recovery rates in culture media of vitamin KO and K were $45 \pm 3\%$ and $99 \pm 0.06\%$, respectively; in cell extract they were $42 \pm 4\%$ and $99 \pm 0.08\%$, respectively. Furthermore, after 10 days of incubation at 37°C in the medium, no spontaneous vitamin K or vitamin KO loss was observed. DL- α -Tocopherol acetate (sigma-aldrich) was used at 5 ppm as internal standard of vitamin K extraction.

Warfarin measurements

Warfarin was extracted on the same sample than vitamin K extraction, from the remaining organic phase after addition of 5mL of diethyl ether. After 10 minutes of centrifugation at 3000 x g, the upper phase was removed and dried under nitrogen gaz. Dry residues were dissolved in 100 μL of methanol and warfarin was quantified by UV-HPLC. The analytical column was an Xbridge RP18 column, 130Å, 3.5 μm , 4.6 mm X 150 mm (Thermofisher, France) and the UV detector (L-4250 UV-VIS Detector) was used at $\lambda = 300\text{nm}$. The mobile phase was composed of 60% of H_3PO_4 in water (0.2 %) - 40% acetonitrile (flow rate = 1 mL/minute at 30°C). The recovery rates of warfarin from medium or cell extracts were 100%. Warfarin concentration was determined in cell assuming a HEK293 volume of about 1700fL (measured by cell counter).

Construction of mammalian expression vectors and transgenic cell line generation

Human HEK293 cell line was used to generate PCR products for VKORC1 (from start to stop codon) and PDI, (from signal peptide to stop codon) fused with a c-myc tag (EQKLISEEDL) via flexible spacer (GGSGGSGGS) at C-terminus end. PCR products were digested by BamH1 and Xba1 restriction enzymes (Takara Bio, France) and introduced into the mammalian expression vector pcDNATM3.1(+) containing geneticin resistance (Invitrogen, France). All constructions were used to produce a stable cell line. Cells were transformed with Lipofectamine 2000TM (Invitrogen, France) according to commercial instructions and selected for stable expression during four weeks with GeneticinTM (Gibco, France). Isolation of VKORC1 monoclonal cell was performed after adequate dilution in cell culture dish, by picking clones with PYREXTM cloning cylinders (fisher scientific, France).

Measurement of cell protein expression by western blotting

After protein expression, cell culture media was removed, adherent cells were rinsed with phosphate-buffered saline and harvested with NP-40 lysis buffer (1% NP-40, 150 mM sodium chloride, 50mM Tris-HCl pH 8.0, 5mM EDTA). Prior to SDS-polyacrylamide gel electrophoresis, cell proteins were denatured in Laemmli buffer. After wet transfer on PVDF immobilon-P transfer membrane (Millipore, France), protein expression was detected by immunoblotting using anti-c-myc tag antibody (Myc Tag rabbit mAB, 71D100 (Ozyme, France)) and anti-GAPDH antibody (G9545 (Sigma, France)) as loading control. Detection antibody was an anti-rabbit HRP-conjugated (Anti Rabbit IgG HRP Linked 7074 (Ozyme, France)). After incubation with ECL RevelBlot Intense (Ozyme, France), chemiluminescence was collected by image densitometry analysis system Pxi (Syngene, France). Signal intensity was quantified using the ImageJ software (<https://imagej.nih.gov/ij/>, 1997-2018).

Statistical analysis

Statistical analysis was performed using R statistical software (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>) and GraphPad Prism Software (San Diego, California USA, www.graphpad.com). IC₅₀ of warfarin was determined by nonlinear regression using most adequate model. Comparisons between cell groups were made using Kruskal-Wallis interaction test follow by a pairwise Wilcoxon rank sum test, with *p value* < 0.05 considered significant (*) and *p value* < 0.01 (**).

Results

Exploration of the K quinone/KO balance after incubation of HEK293 cells in the presence of vitamin K

The K quinone/KO balance was monitored as a function of time in HEK293 cells after adding increasing concentrations of vitamin K₁ quinone in the culture medium. Five different concentrations were tested (10 µM, 25 µM, 50 µM, 70 µM and 150 µM) and three incubation times were performed (6 hours, 24 hours and 48 hours). The K quinone/KO balance was evaluated by measuring the extracellular and intracellular concentrations of vitamin K quinone and vitamin KO. Results are shown in Figure 2. When 10 µM was introduced into the culture medium, regardless of incubation time, vitamin K quinone concentration in cells was unchanged, from 6 to 48 hours after incubation (*p-value* = 0.93 regarding initial concentration) and no intracellular or extracellular accumulation of vitamin KO was detected from 6 to 48 hours after incubation. When 25 µM vitamin K quinone was introduced into the culture medium, the intracellular concentration of vitamin K quinone was unchanged throughout the incubation from 6 hours to 48 hours after incubation (*p-value* = 0.1407 compared to initial concentration), but the concentration of vitamin KO increased in the medium (*p-value* = 0.02)

reaching a plateau (1.5 fold) after 24h of incubation and increased linearly in the cells from 6 hours to 48 hours after incubation (p-value < 0.01 regarding initial concentration). When 50 and 70 μ M vitamin K quinone were added into the medium, the intracellular concentration of vitamin K quinone increased linearly throughout the incubation (respectively p-value = 0.002 and p-value < 0.01), and the intracellular and extracellular concentrations of vitamin KO increased rapidly (intracellularly, p-value < 0.01 at 50 μ M and 70 μ M and extracellularly, p-value < 0.05 at 50 μ M and p-value < 0.01 at 70 μ M), and reached a plateau at 6 hours of incubation (2.5 to 3 fold in the medium ; 1.5 to 2.5 fold in cells). When 150 μ M of vitamin K quinone was added, cell mortality was observed massively after 48 h of incubation.

Warfarin effect on the K quinone/KO balance in HEK293 cells incubated in the presence of vitamin K

The effect of warfarin on the K quinone/KO balance was evaluated by incubating HEK293 cells with increasing amounts of warfarin in the cell media at fixed vitamin K quinone concentration (70 μ M). The uptake of warfarin into the cell was linear ($R^2=0.9837$). Intracellular vitamin K quinone accumulation was approximately four times the extracellular concentration. The addition of warfarin to the medium resulted in an increase of vitamin KO concentration in cells and medium, associated with a decrease of K quinone/KO ratio (Figure 3). This half maximal inhibitory concentration (IC_{50}) was evaluated using various parameters with converging results (from 0.54 ± 0.45 to 2.58 ± 1.56 μ M of warfarin) (Figure 3).

Effect of VKORC1 knock-out or overexpression on the K quinone/KO balance in HEK293 cells incubated in the presence of vitamin K.

The effect of VKORC1 enzyme on the K quinone/KO balance was evaluated by incubating VKORC1- stably overexpressing cells and VKORC1 and VKORC1L1 knocked-out cells, at

fixed vitamin K quinone concentration (70 μ M). In the DKO cell line, significant accumulation of intracellular KO (approximately by a factor of 2) and significant decrease of intracellular K quinone/KO ratio, were observed at the same intracellular uptake of vitamin K quinone compared to other cell lines. No effect was observed for the polyclonal cell line stably overexpressing VKORC1 (Figure 4).

To go further in the analysis of the role of VKORC1 in the K quinone/KO balance, different monoclonal HEK293 cell lines, overexpressing different amounts of VKORC1, were produced. Levels of VKORC1 overexpression were determined by a semi-quantitative western blot analysis. Compared to a reference monoclonal cell line ("monoA"), the other monoclonal cell lines showed a 1.8 to 4-fold higher variation in VKORC1 overexpression ("monoB" = 1.8 ± 0.6 , "monoC" = 2.1 ± 1.0 and "monoD" = 4.0 ± 0.7). Expression level of GGCX between cells were compared by quantitative PCR. Expression level of GGCX was unchanged in VKORC1-overexpressing cells and basal HEK293 cells (data not shown). The K quinone/KO balance was then evaluated under standard conditions (30 hours of incubation at 70 μ M of vitamin K quinone). Although the expression levels of VKORC1 were different between the monoclonal lines, no difference in the K quinone/KO balance was observed (Figure 5).

Effect of PDI overexpression on the K quinone/KO balance in HEK293 cells incubated in the presence of vitamin K₁.

Polyclonal PDI-stably overexpressing cell lines were produced. Expression level of PDI, VKORC1 and GGCX was compared to HEK293 cells by quantitative PCR. Expression level of GGCX and VKORC1 was unchanged, while PDI was increased. The effect of PDI enzyme on the K quinone/KO balance was evaluated by incubating these PDI-overexpressing cells with 70 μ M of vitamin K quinone. Intracellular vitamin K quinone, KO and K quinone/KO balance are presented in Figure 5. The PDI cell line, compared to mock cell lines, presented a

significantly lower intracellular KO concentration after 30 hours of incubation for the same vitamin K uptake (Figure 4B). As a result, intracellular K quinone/KO ratio in the PDI-overexpressing cell line was twice as high as in the mock cell line. Expression level of GGCX was unchanged in VKORC1-overexpressing cells and basal HEK293 cells (data not shown).

Discussion

This study reveals that VKORC1-mediated activities -i.e., VKOR and VR activities- are limited in HEK293 cells, whereas these cells have previously been widely used in cellular approaches for characterization of VKORC1 mutations. This demonstration was obtained using a direct functional cell-based assay whereas previous cell-based assays can be qualified as indirect approaches. Indeed, our cell-based assay is based on the measurement of the K quinone/KO balance at steady state, vitamin KO being the substrate and vitamin K quinone the product of VKORC1 when catalysing the VKOR activity; vitamin K quinone being also the substrate of VKORC1 when catalysing the VR activity. Previous cell-based assays used to characterize VKORC1 activity were based on the measurement of the gamma-carboxylation of a VKDP reporter, a by-product of the vitamin K cycle.

Since the activities catalyzed by VKORC1, *i.e.* VKOR and VR activities, are steps in the vitamin K cycle, it was first necessary to characterize and understand the vitamin K cycle in HEK293 cells. Since the Dulbecco's Modified Eagle Medium used to cultivate HEK 293 cells provides very limited quantity of vitamin K in any form, it was necessary to supplement the medium with vitamin K to ensure that the vitamin K cycle functions. Vitamin K in its quinone form was preferred to the epoxide form, because too much binding of the KO form (40% ± 2.5%) to the surfaces of the culture plates was observed when used in high concentrations, contrary to vitamin K quinone. In addition, our studies revealed an influence of the vitamin K formulation. Injectable vitamin K quinone (used therapeutically) appears to be more

299 bioavailable than vitamin K quinone dissolved in DMSO (data not shown). Anyway, these
300 results demonstrate that cellular assays focusing on the vitamin K cycle must be performed with
301 the same substrate and in the same manner, so that results can be compared between studies.
302 Unfortunately, this has not been the case in previous studies using indirect cells-based assays
303 (Sun et al. 2005; Hallgren et al. 2006; Tie et al. 2011, 2013; Fregin et al. 2013; Czogalla et al.
304 2013; Haque et al. 2014).

305 A first possible point of limitation of the vitamin K cycle was first considered, the entry of
306 vitamin K into the cell (Figure 1 and 2). Our results indicate that the entry of vitamin K quinone
307 is probably facilitated by one or more saturable transport proteins (hyperbolic appearance of
308 the curve). To achieve maximum intracellular concentrations of vitamin K in cells, high
309 concentrations of vitamin K quinone in the medium are required (maximum intracellular
310 concentrations of vitamin K quinone being achieved by adding 130 μ M of vitamin K to the
311 medium), whereas previous assays in VKOR cells were performed with much lower
312 concentrations (5-10 μ M). A significant increase in intracellular vitamin K concentration (100-
313 fold) is observed with the addition of at least 50 μ M vitamin K quinone and is dependent on the
314 incubation time (Figure 2). These concentrations, even those used in the indirect approach, are
315 much higher than the concentrations reported in human liver or plasma (Usui et al. 1990;
316 Thijssen and Drittij-Reijnders 1996). Nevertheless, tissue concentrations of vitamin K may be
317 much lower than the local concentration at the cellular level. In this study, vitamin K/KO
318 balance was measured in the HEK293 or modified HEK cells after an incubation period of 30
319 hours in the presence of 70 μ M of vitamin K quinone, because characterization of the cycle
320 steps must be performed at a saturating substrate to avoid substrate limitation and when steady-
321 state is reached.

322 Since the vitamin K supplied is in the form of quinone, it was essential to evaluate whether the
323 quinone was incorporated into the vitamin K cycle and became a possible substrate for the

324 VKOR activity catalyzed by VKORC1. The vitamin K quinone entering the cell must therefore
 325 first be converted into vitamin KH₂ by the VR activity catalyzed in part by VKORC1 but also
 326 by at least one other enzyme not identified at this stage, in order to be used subsequently by
 327 GGCX catalyzing the gamma carboxylation reaction. Under the action of GGCX, vitamin KO
 328 is produced in the presence of a VKDP. Vitamin KO is then converted back to vitamin K
 329 quinone via the VKOR activity catalyzed exclusively by VKORC1. In our HEK293 cells, after
 330 addition of vitamin K quinone into the medium, the vitamin K quinone/KO balance is always
 331 at the steady-state in favor of vitamin K quinone. Indeed, after addition of 70 μM of K quinone
 332 to the medium and incubation for 30 hours, the vitamin KO represents about 1.5% of the total
 333 vitamin K forms (K+KO), nevertheless in a dynamic system in which KO is immediately
 334 transformed back into K and vice versa. The addition of vitamin K quinone to the incubation
 335 medium does generate vitamin KO, but in limited quantities. The limitation of the vitamin K
 336 cycle could be due to VR and/or VKDC activities. To decide between the 2 solutions is complex
 337 because of the non-measurement of vitamin KH₂. KH₂ being very unstable, in the presence of
 338 oxygen, it is immediately converted back to vitamin K quinone. Nevertheless, the fact that in
 339 the further study we were able to modify the vitamin K/KO balance by increasing VKOR
 340 activity while GGCX expression was not modified, suggests that VKDC activity is not limiting.
 341 VR activity is probably the limiting step in the vitamin K cycle in HEK293 cells. In other cells,
 342 the limiting steps are probably different. Indeed, the K quinone/KO balance seems to be largely
 343 influenced by the cell type. In the human liver lines Hep3B and Huh7 and in the human
 344 osteoblast line MG-63, the K quinone/KO equilibrium is lower than in HEK 293 cells (Figure
 345 S1 in the supplemental data) due to a higher accumulation of vitamin KO. Therefore,
 346 comparison of results obtained in cell systems require that they are obtained in the same cell
 347 system. Indirect cell-based assays already published in the literature are performed in a variety
 348 of cell types with different enzymatic equipment, so comparison of results must be cautious.

Due to the limitation of the vitamin K cycle by the VR activity in HEK293 cells, does the use of this cell line allow the proper characterization of VKOR activity? For this purpose, we used warfarin, a specific inhibitor of VKOR activity. In the presence of warfarin, we observed in the cell and in the medium, a significant increase in vitamin KO concentrations and a decrease in the K/KO ratio. Although VKORC1, and to a lesser extent VKORC1L1, are inhibited, vitamin K hydroquinone is still produced by VR activity and utilized by the GGCX enzyme. However, because VKOR is inhibited by warfarin, there is an accumulation of vitamin KO. As demonstrated in other studies, VR activity appears to be resistant to warfarin, demonstrating the existence of another enzyme catalyzing VR activity as previously proposed (Tie et al. 2011). The concentration of vitamin KO, or the ratio of vitamin K quinone and KO concentrations, are therefore excellent parameters for assessing VKOR activity in HEK293 cells. The IC₅₀ of warfarin towards VKOR activity, based on the measurement of these parameters in the cell or in the medium, varies from 0.54 μ M to 2.58 μ M (taking into account the cell volume). This IC₅₀ is similar to that reported for recombinant human VKORC1 expressed in the yeast *Pichia pastoris* using the DTT-driven VKOR assay (Hodroge et al. 2012), but significantly higher than those reported by indirect cellular assays overexpressing human VKORC1 (Tie et al. 2013; Czogalla et al. 2016). Nevertheless, these published IC₅₀s were based on the concentration of warfarin added to the medium (apparent IC₅₀), not on the measured intracellular concentrations of warfarin, which may totally explain the difference. Determination of the apparent IC₅₀ therefore undoubtedly allows comparison of the sensitivity of VKOR activity to different inhibitors (if the intracellular concentrations of the latter are determined) and characterization of the impact of human or rodent VKORC1 mutations on VKA sensitivity (only if the endogenous VKOR enzymes are neutralized). However, can cell-based systems (direct or indirect) using the HEK293 cell line characterize the velocity of VKOR activity? To answer this question, we developed different monoclonal cell lines overexpressing VKORC1.

374 Surprisingly, the K quinone/KO balance is not modified by increasing VKORC1 expression
 375 (Figure 4 and 5), while the overexpressed VKORC1 is definitively functional. Indeed, to
 376 confirm the functionality of overexpressed VKORC1, microsomal fractions of HEK293 cells
 377 and HEK293 cells overexpressing VKORC1 were prepared and basal VKOR activity was
 378 measured in the presence of DTT. VKORC1-overexpressing cells showed a lineage-dependent
 379 increase in VKOR activity of at least 3-fold (data not shown). This suggests a limitation of
 380 VKORC1-mediated activities in HEK293 cells. A previous study published by Hallgren et al.
 381 in 2006 reported the same observation (Hallgren et al. 2002). Therefore, VKORC1 activation
 382 might be limiting for VKOR/VR activities in HEK293 cells. In fact, VKORC1, requires prior
 383 activation to reduce vitamin KO to K and K to KH₂. It has been proposed that this activation
 384 depends on a protein partner from the protein disulfide isomerase family and the conserved
 385 luminal cysteines 53 and 51 of VKORC1. Although several candidates have been proposed, no
 386 consensus has been reached on the exact identity of this partner (Preusch 1992; Schulman et al.
 387 2010; Schaafhausen et al. 2011). We overexpressed PDI, the most frequently proposed
 388 candidate in the literature in the baseline HEK293 cell line. Overexpression of PDI leads to a
 389 shift in cell equilibrium in favor of VKOR activity (less vitamin KO at steady state), with a
 390 vitamin K quinone/KO ratio of up to 2 (Figure 4). This is the first functional evidence that PDI
 391 is a redox activator of VKORC1. Further experiments will be needed to determine whether
 392 other potential redox partners (TMX1 and TMX4) can also be functionally considered as redox
 393 partners of VKORC1. These results provide evidence that VKORC1 is a limited step restricted
 394 in HEK293 cells by its protein partner. Increasing the expression of VKORC1 in HEK293 cells,
 395 therefore, may have no or little effect, as the VKORC1 enzyme is already functioning in-vivo
 396 at under-regime. This limitation may hinder characterization of the functional consequences of
 397 VKORC1 mutations on enzyme velocity, which may explain the differences in VKORC1
 398 results between DTT-driven and cell-based assays.

399

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404 Authorship Contributions:

405 Data collection from the cell-based functional assay: T.C; Data analysis and figures production:

406 T.C and V.Lattard; Vitamin K quantification: E.B; Experimental design: T.C, E. B, V.Lambert

407 and V.Lattard. Writing-Original draft: T.C. and V. Lattard; Supervision: V.Lattard and

408 V.Lambert; Project administration: V.Lattard; Funding acquisition: V.Lattard.

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413

414 Conflict of interest statement:

415 The authors declare no competing financial interests.

416

417 Data sharing:

418 The data that support the findings of this study are available on request from the corresponding

419 author.

420

421 Abbreviations:

422 **DKO:** Double knock out VKORC1 and VKORC1L1

423 **PDI:** Protein disulfide isomerase

424 **K:** Vitamin K quinone
 425 **KH₂:** Vitamin K hydroquinone
 426 **KO:** Vitamin K epoxide
 427 **VKA:** Vitamin K antagonist
 428 **VKDC:** Vitamin K dependent carboxylation
 429 **VKOR:** Vitamin K epoxide reductase
 430 **VKR:** Vitamin K reductase

431

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Legends of figures:

Figure 1: Vertebrate cell vitamin K pathway. Enzymes of the vitamin K cycle are represented in their natural environment (ie: endoplasmic reticulum membrane bilayer (ER

membrane) and lumen (ER lumen)). The four main enzymes of the vitamin K cycle (gamma-glutamyl carboxylase (GGCX), vitamin K epoxide reductase complex subunit 1 (VKORC1), vitamin K epoxide reductase complex subunit 1-like enzyme 1 (VKORC1L1), NAD(P)H dehydrogenase quinone 1 (NQO1) contribute to establishing vitamin K equilibrium. Vitamin K transport (KO = vitamin K epoxide, K = vitamin K quinone and KH₂ = vitamin K hydroquinone) across membranes is indicated by red arrows. Supposed VKORC1 and VKORC1L1 reduced activation by protein disulfide isomerase-like proteins (PDIs) system could constitute a limiting step for vitamin K cycle. Metabolism is represented by cytochrome P450 (CYP450).

Figure 2. Vitamin K quinone and KO as a function of time in HEK293 cells after adding vitamin K₁ in the culture medium. (A) and (B) illustrate the evolution of intracellular (for 10⁶ cells) and extracellular concentrations of vitamin K epoxide (KO) depending on the concentration of vitamin K quinone added in cell media expressed in fold change relative to T0 (without vitamin K added in the media). (C) and (D) illustrate the evolution of the intracellular (for 10⁶ cells) or extracellular concentrations of vitamin K quinone (K) depending on the concentration of vitamin K quinone added in the cell media expressed in fold change relative to T0 (without vitamin K added in the media). At T0, vitamin K concentration in medium was 3.31±0.09 nM for 10⁶ cells; vitamin K concentration in cells was 22.3±0.02 nM for 10⁶ cells; vitamin KO concentration in medium was 4.20±0.8 nM for 10⁶ cells; vitamin KO concentration in cells was 0.33±1.04 nM for 10⁶ cells. Values indicated the mean ± standard deviations of two or three individual determinations.

Figure 3. Effect of warfarin on vitamin K equilibrium in HEK293 cells. HEK293 cells were incubated for 30 hours in the presence of increasing concentrations of warfarin added in the cell media at 70 μ M of vitamin K quinone. (A) The intracellular concentration of warfarin ($\blacktriangle$) as a function of warfarin concentration added in the cell media, a linear model was applied and plot in full purple line (—). (B), (C), (D), and (E) Intracellular or extracellular vitamin KO ($\bullet$) or ratio between vitamin K quinone and vitamin KO ($\blacksquare$) as a function of intracellular warfarin concentration. Four-parameters dose-response model was applied and plot in full red or green line (— or —). Values are the mean $\pm$ SEM of two or three individual determinations. The concentrations of vitamin K quinone in the medium and cells were measured, and the concentrations were unchanged whatever the concentration of warfarin (data not shown).

Figure 4. Effect of knock out or overexpression of VKORC1 or PDI on the K quinone/KO balance in HEK cells: DKO (double Knock-out of VKORC1 and VKORC1L1), VKORC1 (polyclonal cell line stably overexpressing VKORC1), PDI (polyclonal cell line stably overexpressing PDI) compared to Mock cell line (HEK293), after 30 hours of incubation at 37°C with 70 μ M of vitamin K quinone. The concentration of vitamins KO in (A) ($\bullet$), K quinone in (B) ($\blacktriangle$) and the ratio of both in (C) ($\blacksquare$) were determined by LC-MS/MS in cell extracts. Results are normalized to the HEK293 cell line. The red dashed line highlights the mean level of HEK293 cell line equilibrium. (D) Expression or extinction of proteins (VKORC1 and PDI) was confirmed, equivalent amounts of protein lysates (40 μ g) were loaded on SDS PAGE and analyzed in a western blot using a polyclonal c-Myc antibody.

Figure 5. K quinone/KO balance in different monoclonal HEK cell lines overexpressing VKORC1 at different levels (monoA to monoD). Cells were incubated for 30 hours at 37°C

695 with 70 μ M of vitamin K quinone. Vitamin intracellular concentration KO in (A) (●), K quinone
696 in (B) (▲), and the ratio of both in (C) (■) were determined by LC-MS/MS. Results are
697 presented as fold change compared to the “monoA” monoclonal cell line. The red dashed line
698 highlights the mean level of the “monoA” cell line.
699 (D) Expression of VKORC1 was confirmed and quantified. Equivalent amounts of protein
700 lysates (40 μ g) from four monoclonal cell lines (monoA to D), were analyzed in a western blot
701 using a polyclonal c-Myc antibody.

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Figure 1: Vertebrate cell vitamin K pathway. Enzymes of the vitamin K cycle are represented in their natural environment (ie: endoplasmic reticulum membrane bilayer (ER membrane) and lumen (ER lumen)). The four main enzymes of the vitamin K cycle (gamma-glutamyl carboxylase (GGCX), vitamin K epoxide reductase complex subunit 1 (VKORC1), vitamin K epoxide reductase complex subunit 1-like enzyme 1 (VKORC1L1), NAD(P)H dehydrogenase quinone 1 (NQO1) contribute to establishing vitamin K equilibrium. Vitamin K transport (KO = vitamin K epoxide, K = vitamin K quinone and KH₂ = vitamin K hydroquinone) across membranes is indicated by red arrows. Supposed VKORC1 and VKORC1L1 reduced activation by protein disulfide isomerase-like proteins (PDIs) system could constitute a limiting step for vitamin K cycle. Metabolism is represented by cytochrome P450 (CYP450).

300x300mm (71 x 71 DPI)

Figure 2. Vitamin K quinone and KO as a function of time in HEK293 cells after adding vitamin K1 in the culture medium. (A) and (B) illustrate the evolution of intracellular (for 106 cells) and extracellular concentrations of vitamin K epoxide (KO) depending on the concentration of vitamin K quinone added in cell media expressed in fold change relative to T0 (without vitamin K added in the media). (C) and (D) illustrate the evolution of the intracellular (for 106 cells) or extracellular concentrations of vitamin K quinone (K) depending on the concentration of vitamin K quinone added in the cell media expressed in fold change relative to T0 (without vitamin K added in the media). At T0, vitamin K concentration in medium was 3.31 ± 0.09 nM for 106 cells; vitamin K concentration in cells was 22.3 ± 0.02 nM for 106 cells; vitamin KO concentration in medium was 4.20 ± 0.8 nM for 106 cells; vitamin KO concentration in cells was 0.33 ± 1.04 nM for 106 cells. Values indicated the mean $\pm$ standard deviations of two or three individual determinations.

338x190mm (96 x 96 DPI)

Figure 3. Effect of warfarin on vitamin K equilibrium in HEK293 cells. HEK293 cells were incubated for 30 hours in the presence of increasing concentrations of warfarin added in the cell media at 70 μM of vitamin K quinone. (A) The intracellular concentration of warfarin (purple triangle) as a function of warfarin concentration added in the cell media, a linear model was applied and plot in full purple line (—). (B), (C), (D), and (E) Intracellular or extracellular vitamin K^o (red circle) or ratio between vitamin K quinone and vitamin K^o (green square) as a function of intracellular warfarin concentration. Four-parameters dose-response model was applied and plot in full red or green line (— or —). Values are the mean ± SEM of two or three individual determinations.

197x260mm (300 x 300 DPI)

Figure 4. Effect of knock out or overexpression of VKORC1 or PDI on the K quinone/KO balance in HEK cells: DKO (double Knock-out of VKORC1 and VKORC1L1), VKORC1 (polyclonal cell line stably overexpressing VKORC1), PDI (polyclonal cell line stably overexpressing PDI) compared to Mock cell line (HEK293), after 30 hours of incubation at 37°C with 70μM of vitamin K quinone. The concentration of vitamins KO in (A) (red circle), K quinone in (B) (blue triangle) and the ratio of both in (C) (green square) were determined by LC-MS/MS in cell extracts. Results are normalized to the HEK293 cell line. The red dashed line highlights the mean level of HEK293 cell line equilibrium. (D) Expression or extinction of proteins (VKORC1 and PDI) was confirmed, equivalent amounts of protein lysates (40μg) were loaded on SDS PAGE and analyzed in a western blot using a polyclonal c-Myc antibody.

83x100mm (254 x 254 DPI)

Figure 5. K quinone/KO balance in different monoclonal HEK cell lines overexpressing VKORC1 at different levels (monoA to monoD). Cells were incubated for 30 hours at 37°C with 70 μ M of vitamin K quinone. Vitamin intracellular concentration KO in (A) (red circle), K quinone in (B) (blue triangle), and the ratio of both in (C) (green square) were determined by LC-MS/MS. Results are presented as fold change compared to the "monoA" monoclonal cell line. The red dashed line highlights the mean level of the "monoA" cell line. (D) Expression of VKORC1 was confirmed and quantified. Equivalent amounts of protein lysates (40 μ g) from four monoclonal cell lines (monoA to D), were analyzed in a western blot using a polyclonal c-Myc antibody.

338x250mm (63 x 63 DPI)