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Two enzymes catalyze vitamin K 2,3-epoxide reductase activity in mouse: VKORC1 is highly expressed in exocrine tissues while VKORC1L1 is highly expressed in brain

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ABSTRACT

VKORC1 and VKORC1L1 are enzymes that both catalyze the reduction of vitamin K2,3-epoxide via vitamin K quinone to vitamin K hydroquinone. VKORC1 is the key enzyme of the classical vitamin K cycle by which vitamin K-dependent (VKD) proteins are γ-carboxylated by the hepatic γ-glutamyl carboxylase (GGCX). In contrast, the VKORC1 paralog enzyme, VKORC1L1, is chiefly responsible for antioxidative function by reduction of vitamin K to prevent damage by intracellular reactive oxygen species. To investigate tissue-specific vitamin K 2,3-epoxide reductase (VKOR) function of both enzymes, we quantified mRNA levels for *VKORC1*, *VKORC1L1*, *GGCX*, and *NQO1* and measured VKOR enzymatic activities in 29 different mouse tissues. *VKORC1* and *GGCX* are highly expressed in liver, lung and exocrine tissues including mammary gland, salivary gland and prostate suggesting important extrahepatic roles for the vitamin K cycle. Interestingly, VKORC1L1 showed highest transcription levels in brain. Due to the absence of detectable NQO1 transcription in liver, we assume this enzyme has no bypass function with respect to activation of VKD coagulation proteins. Our data strongly suggest diverse functions for the vitamin K cycle in extrahepatic biological pathways.

Introduction

Vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) is the key enzyme of the vitamin K cycle essential for post-translational modification of vitamin K-dependent proteins (VKD proteins) [1,2]. VKD proteins are modified by the γ-glutamyl carboxylase (GGCX) which converts specific glutamic acid residues to γ-glutamyl dicarboxylic acid (Gla) residues. This modification confers VKD proteins with their biological activities. Simultaneously, GGCX oxidizes vitamin K hydroquinone to vitamin K 2,3-epoxide. In turn, VKORC1 subsequently reduces vitamin K 2,3-epoxide back to the quinone and hydroquinone forms, completing a vitamin K redox cycle known as the "vitamin K cycle" [3].

The paralog enzyme of VKORC1, VKORC1-like 1 (VKORC1L1), also functions both as a vitamin K 2,3-epoxide reductase and as a vitamin K quinone reductase, but has lower affinity for K vitamins compared to that of VKORC1 [4]. Additionally, VKORC1L1 appears not to be capable of driving the classical vitamin K cycle to adequately produce activated VKD clotting factor proteins in VKORC1 knock-out mice [5,6]. However, during experimentally-induced oxidative stress, *VKORC1L1* mRNA is significantly upregulated *in vitro*, suggesting its function in antioxidative pathways as protective against free radicals [4].

Since the 1980s, it has been speculated that NAD(P)H:quinone oxidoreductase 1 (NQO1) might function as a bypass enzyme in the vitamin K cycle in cases of 4-hydroxycoumarin intoxication where large doses of vitamin K have been known to be antidotal [7]. Compared to VKORC1, NQO1 is several orders of magnitude less sensitive to 4-hydroxycoumarin inhibition and catalyzes the reduction of vitamin K quinone only at high, non-physiological vitamin K concentrations *in vitro* [8–10]. However, recent studies concluded that NQO1 is not responsible for γ-carboxylation of VKD proteins in the classical vitamin K cycle in *in vitro* cell studies as well as in NQO1-deficient mice [11,12,7].

Apart from the well-established functions of VKORC1 and GGCX in the vitamin K cycle, including enabling haemostatic pathways by γ-carboxylation of VKD coagulation factors (*e.g.* FII, FVII, FIX, FX), their

Abbreviations: DTT, dithiothreitol; Gas6, Growth arrest-specific protein 6; GGCX, γ-glutamyl carboxylase; Gla, γ-glutamyl dicarboxylic acid; MGP, matrix Gla-protein; NQO1, NAD(P)H:quinone oxidoreductase 1; VKD, vitamin K-dependent; VKOR, vitamin K 2,3-epoxide reductase; VKORC1, vitamin K 2,3-epoxide reductase complex subunit 1; VKORC1L1, VKORC1-like 1.

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functions in extrahepatic tissues are less well investigated. Also, other VKD proteins with a wide diversity of non-haemostatic functions have been reported. For example, matrix Gla-protein (MGP) and osteocalcin are involved in calcium regulation and bone homeostasis. MGP was found to act as a calcification inhibitor *in vivo* [13]. Significantly higher rates of vascular calcification and associated aortic rupture were observed in MGP-knockout mice [13]. Osteocalcin is a physiological marker for osteoblast activity and bone formation and plays an important role in regulating bone metabolism [14–16]. Recent animal studies suggest an additional connection between bone remodeling and systemic metabolism as osteocalcin-deficient mice are hyperglycemic and hypoinsulinemic [17]. Another VKD protein, growth arrest-specific protein 6 (Gas 6), is implicated in the regulation of multiple cellular functions including growth, anti-apoptotic pathways, cell adhesion, and chemotaxis [18–21]. The VKD protein periostin was found to play a role in prenatal heart development [22]. Additionally, biological functions are not known for additionally discovered VKD proteins including PRGP1 through PRGP4 [23–27].

The present study aims to provide more insight into the role of the vitamin K cycle in extrahepatic tissues by examining mRNA levels of the vitamin K cycle-related enzymes *VKORC1*, *VKORC1L1*, *GGCX* and *NQO1* and the vitamin K 2,3-epoxide reductase (VKOR) activity in 29 distinct mouse tissues.

Materials and Methods

Mouse Tissue Preparation

Microsomes and mRNA were prepared from various tissues of 7 week-old male and female CD1 wild-type mice (five of each sex; Charles River Laboratories International, Sulzfeld, Germany). Mice were sacrificed and 29 different tissues were dissected including (1) adrenal gland, (2) bone, (3) brain, (4) caecum, (5) colon, (6) diaphragm, (7) duodenum, (8) eye, (9) heart, (10) kidney, (11) liver, (12) lung, (13) lymph node, (14) mammary gland, (15) masseter muscle, (16) muscle, (17) oesophagus, (18) pancreas, (19) salivary gland, (20) skin, (21) soft tissue, (22) stomach, (23) spleen, (24) tongue, (25) vessels, (26f) ovary, and (27f) uterus from females, and (26 m) testis and (27 m) prostate from males. The excised tissues were snap-frozen in liquid nitrogen and stored at -80 °C until use.

Preparation of mRNA and Real Time PCR

Isolation of mRNA from excised tissues was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Reverse transcription of mRNA was performed using the QuantiTect Reverse Transcription-Kit (Qiagen) in conjunction with oligo-dT primers and hexanucleotides. Real-time PCR was performed using 2 μl of cDNA and the ABsolute QPCR Mix including ROX (Thermo Fisher, Hamburg, Germany). For the preparation of standards, cDNAs of *VKORC1*, *VKORC1L1*, *GGCX*, *NQO1* and hydroxymethylbilanesynthase (*HMBS*, a housekeeping-gene) were cloned into pGEM-T vectors using the pGEM-T Easy vector system kit (Promega, Madison, USA). Since exocrine pancreas produces high concentrations of RNase, this tissue was excluded from mRNA quantification experiments. For direct comparison of *VKORC1* and *VKORC1L1* transcription, both sequences were cloned in tandem into pGEM-T vectors and used as standards for examination of liver and brain tissues. Standard curves for real-time PCR experiments were prepared by serial dilutions of the vectors to yield one to 10^7 copies. *VKORC1*, *VKORC1L1*, *GGCX* and *NQO1* transcription levels were calculated according to the respective standard curves and normalized to determined constitutive *HMBS* transcription levels. Probes were labeled with FAM at the 5´-prime end and with a black-hole-quencher (BHQ) at the 3´-prime end. Real-time PCR was performed using the ABI Prism 7700 Sequence detection system. All assays were performed in duplicate. Primer sequences are listed in the online supplementary file.

Preparation of Microsomes

Frozen tissue samples were mechanically disrupted and homogenised in 500 μL ice cold buffer A (250 mM sucrose, 25 mM imidazol, 80 mM KCl, pH 7.6) [28]. To reduce protease activity, a protease inhibitor (Complete Mini, Roche Applied Science, Mannheim, Germany) was added to buffer A. The following tissues were pooled (male and female samples separately) due to the small amounts of sample material: adrenal gland, bone, ceacum, colon, diaphragm, eye, mammary gland, oesophagus, salivary gland, skin, aorta, vessels, tongue, masseter muscle, lymph node, ovary, uterus, duodenum, testis, prostate. All other tissues were analyzed for each mouse as individual samples. Sample homogenates were centrifuged at 10,000 xg for 10 min to remove mitochondria and cellular debris. Microsomes were isolated from supernatants by ultracentrifugation at 100,000 xg for 1 h. After washing the microsome pellet in buffer B (25 mM imidazol, 0.5% CHAPS, pH 7.6) and centrifugation at 100,000 xg for 1 h, the microsomal pellets were resuspended in 150 μL buffer B and stored at -80 °C until further use. Storage was not longer than 3 days prior to performing activity measurements.

DTT-driven Vitamin K 2,3-epoxide Reductase (VKOR) Activity Assay

VKOR enzymatic activity was measured using the standard dithiothreitol (DTT)-driven method according to published procedures [1, 29]. Mircrosomes (30 μL) were mixed together with 459 μL buffer B and 20 μL 125 mM DTT (5 mM final concentration in the assay) on ice. After incubation for 1 min at room temperature, 5 μL of 400 mM $CaCl_2$ and 16 μL of 1.656 mM vitamin K_1 2,3-epoxide in EtOH were added. Reaction was stopped after incubation for 1 h at 30 °C by addition of 1 mL isopropanol:hexane (3:2 v/v). Vitamin K_2 2,3-epoxide was used in the organic solvent mixture as internal standard (10 μl of 50 μM). After organic phase extraction, a fixed volume of solvent phase was removed (300 μl), vacuum-dried and resuspended in isopropanol:methanol (1:4 v/v), and resolved by HPLC chromatography. Quantitation of K vitamers was done by integration of absorbance peaks (248 nm) and compared to 150 μM vitamin K_2 standard peaks to calculate recovery efficiencies.

VKOR activity was calculated as the ratio of vitamin K_1 2,3-epoxide converted to vitamin K_1 quinone per time, normalized to total protein concentration for each sample measured by the Lowry protein assay [30]. Vitamin K epoxidation was performed by oxidation of vitamin K quinone (Sigma-Aldrich) using H_2O_2 according to Tishler *et al.* [31].

Results

Determination of VKORC1, VKORC1L1, GGCX and NQO1 Transcription Levels

Transcription levels of *VKORC1, VKORC1L1, GGCX and NQO1* were investigated by real-time PCR of mRNA preparations from 29 different CD1 wild-type mouse tissues. Presence of *VKORC1* mRNA transcripts could be detected in all tissues examined (Fig. 1A). Highest transcription of *VKORC1* mRNA (mean value 4.79 AU (arbitrary units)) was observed for liver. In glandular tissues, high transcription levels of *VKORC1* were found in salivary gland (2.84 AU) and mammary gland (2.10 AU). In contrast, transcription in adrenal gland was much lower (1.00 AU). All other tissues examined (with exceptions of prostate 2.71 AU and lung 2.51 AU) exhibited transcription values lower than 2.00 AU (Fig. 1A).

With the exception of skin, we found *GGCX* transcribed in all tissues examined with the highest level in liver (0.45 AU), followed by lung (0.28 AU; Fig. 1B). Preparations from testis showed low *GGCX* transcription levels close to the detection limit. For all other tissues, we found intermediate transcription levels ranging from 0.05 to 0.2 AU.

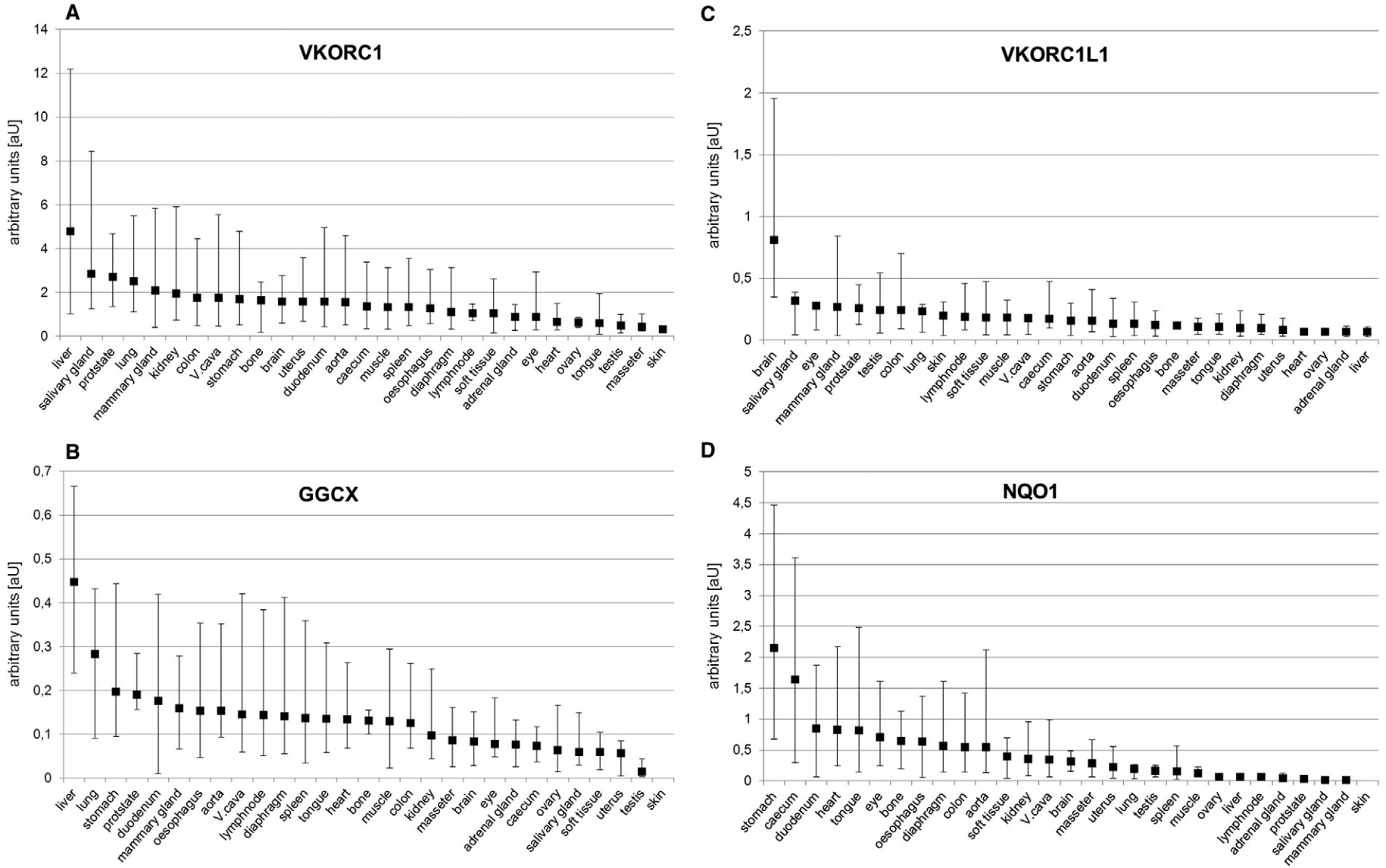

Fig. 1. mRNA expression of *VKORC1*, *GGCX*, *VKORC1L1* and *NQO1* in 26 different mouse tissues. mRNA expression was evalutated of tissues of each 5 male and 5 female mice by two-step real-time PCR in duplicate. As standard the respective cDNA cloned in pGEM-T vector was used in serial dilutions of one to 10^7 copies. Hydroxymethylbilanesynthase (*HMBS*) was used as housekeeping-gene. Mean expression levels are represented by arbitrary units (AU). Each tissue investigated is labeled at the bottom. Error bars show the range of highest and lowest measurements representing interindividual differences in mice. Panel A show mRNA expression levels for *VKORC1*, Panel B transcription levels for *GGCX*, Panel C transcription levels for *VKORC1L1*, Panel D transcription levels for *NQO1*.

Ubiquitous transcription of *VKORC1L1* was found in all tissues examined. The highest transcription level of *VKORC1L1* was detected in brain (0.8 AU; Fig. 1C). In all other tissues, we found transcription levels of *VKORC1L1* ranging from 0.1 to 0.4 AU with lowest the lowest level in liver.

NQO1 showed highest transcription in gastrointestinal tissues (*e.g.*, stomach 2.15 AU, caecum 1.65 AU). Lowest mRNA levels (≤0.1 AU) were detected in liver, glandular tissue, ovar and prostate (Fig. 1D).

Absolute quantitative comparison regarding *VKORC1* and *VKORC1L1* transcription using a *VKORC1/VKORC1L1* bicistronic standard probe vector for liver and brain samples revealed that *VKORC1* transcription in liver was as high as *VKORC1L1* transcription in brain (Fig. 2), suggesting VKORC1L1 has a more significant function in brain, while VKORC1 has the dominant function in liver. The transcription level of *VKORC1L1* in liver represents 2% relative to that of *VKORC1*, whereas *VKORC1* transcription level is 50% relative to that of *VKORC1L1* in brain (Fig. 2).

VKOR Activity Measurements in Microsomes

In the present study, we measured VKOR activity in microsomal preparations from 29 different mouse tissues. Presence of VKOR activity was detected in all tissues examined (Fig. 3). Even though most tissues showed basal level activities, high values were found for liver, pancreas, ovary, uterus, mammary gland, vascular tissue and salivary gland (>1 ng K_1/mg total protein/h). As expected, high activity was found for liver (1.92 ng K_1/mg total protein/h). Interestingly, pancreas showed a comparable value (1.94 ng K_1/mg total protein/h). Furthermore, kidney, spleen, heart and lung showed activity values ranging from 0.40 to 0.89 ng K_1/mg total protein/h. Activity measurements in brain revealed comparably low values with mean activity of ≤0.57 ng K_1/mg total protein/h.

In glandular tissue, we found a gender-specific high VKOR activity for female tissues, relative to that for male tissues, including 1.97 ng K_1/mg total protein/h for mammary gland and 1.82 ng K_1/mg total protein/h for salivary gland (Fig. 4A). In comparison, activity levels in male tissues reached only 0.58 ng K_1/mg total protein/h for mammary gland and only 0.29 ng K_1/mg total protein/h for salivary gland. Contrastingly, adrenal gland showed a mean activity of 0.72 ng K_1/mg total protein/h without significant gender-specific difference.

In gastrointestinal tissue, higher activities were found for oesophagus, stomach and colon. Only basal activity was found for duodenum and caecum. Very high VKOR activities for uterus (1.43 ng K_1/mg total protein/h) and ovary (1.68 ng K_1/mg total protein/h) were found. In comparison, male sex organs showed lower mean activities ranging from 0.39 ng K_1/mg total protein/h in prostate to 0.59 ng K_1/mg total protein/h in testis (Fig. 4B).

Furthermore, we investigated four muscle samples from different origins. Samples from quadriceps femoris, diaphragm and tongue revealed equal mean activity levels of 0.61 ng K_1/mg total protein/h. Samples from masseter exhibited slightly lower activities than for other muscles (Fig. 3).

Because of relatively low availability of tissue quantities, vena cava and aorta were separately pooled with respect to gender. With a total of 0.67 ng K_1/mg total protein/h, we found significantly lower activities for male mice compared to 1.46 ng K_1/mg total protein/h for females. In further pooled tissues from bone, eye, skin, and soft tissue, basal activity levels could be detected in the range of 0.34 to 0.51 ngK_1/mg total protein/h without gender-specific differences. Samples from lymph node showed activities of 0.97 ng K_1/mg total protein/h for both genders.

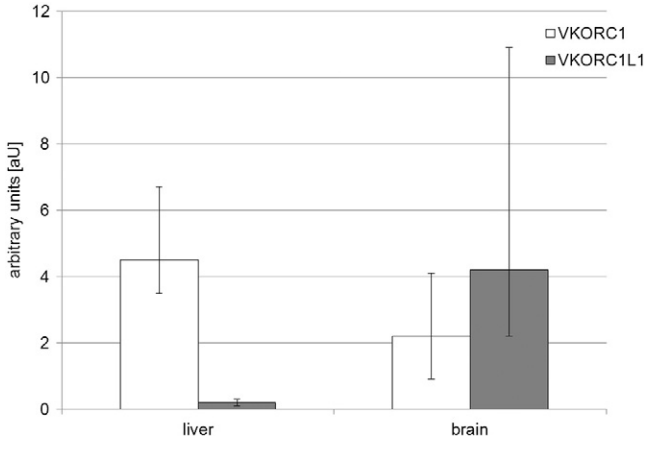

Fig. 2. Quantitative comparison of mRNA transcription levels for *VKORC1* and *VKORC1L1* in liver and brain. To directly compare mRNA transcription levels of *VKORC1* and *VKORC1L1* a standard was used in which both cDNA sequences were cloned into pGEM-T vector. The extent of mRNA expression for *VKORC1* (white bars) and *VKORC1L1* (grey bars) is shown for liver and brain as arbitrary units (AU). Error bars represents the highest and lowest measurement.

Discussion

The vitamin K cycle in liver is crucial to haemostasis. Additionally, it also plays an essential role in extrahepatic tissues. Previous studies demonstrated that vitamin K and the vitamin K cycle are involved in cell growth, inflammation, apoptosis, and Ca^{2+} metabolism, and are associated with diseases that include osteoporosis, cancer, diabetes and vascular calcification [3,13]. These diverse functions are mediated by a number of non-coagulation VKD proteins that include MGP, osteocalcin, Gas6, periostin, the proline-rich Gla proteins (PRGPs), as well as possibly others not yet identified VKD proteins [13,32,33,23]. In addition, a secondary vitamin K pathway is apparently involved in intracellular antioxidation, for which VKORC1L1 generates vitamin K hydroquinone to combat intracellular ROS [4].

In the present study, we focused on the role of known enzymes of the vitamin K cycle. Therefore, we analysed mRNA transcription levels for *VKORC1, VKORC1L1, GGCX,* and *NQO1* in 29 different mouse tissues (Fig. 1). We also investigated VKOR activity in all studied tissues by use of the DTT-driven VKOR assay in order to ascertain relative contributions from the VKORC1 and VKORC1L1 paralog enzymes. Limitation of the DTT-driven VKOR assay is that VKOR activity is determined for both VKOR enzymes. Therefore, we cannot distinguish between the activity of the isozymes VKORC1 and VKORC1L1. There is also no specific inhibitor available to inhibit only one of these enzymes.

As expected, highest mRNA transcription for *VKORC1* and *GGCX* was found in liver, the primary organ of VKD coagulation factor synthesis (Fig. 1A and B). Contrastingly, *VKORC1L1* is transcribed in liver at only 2% relative to the level of *VKORC1* (Fig. 2). This is not surprising because VKORC1L1 is currently percieved to chiefly function in intracellular redox homeostasis [4]. However, recently published data by Tie *et al.* confirmed the ability of VKORC1L1 to convert vitamin K 2,3-epoxide to vitamin K quinone *in vitro* in a *VKORC1* knock-out HEK293 cell line. They further demonstrated that VKORC1L1-mediated VKOR activity was sufficient for γ-carboxylation of an engineered chimeric reporter protein [12]. Accordingly, Tie *et al.* concluded that VKORC1L1 is likely not a crucial enzyme for vitamin K epoxide reduction *in vivo* because it can not rescue the *post partum* fatal bleeding phenotype of *VKORC1*$^{-/-}$ mice under physiological conditions [5,12]. Our findings suggest that VKORC1L1 cannot efficiently bypass VKORC1 functional deficiency in liver due to its low expression level (2% that of VKORC1; Fig. 2). In addition, Hammed *et al.* convincingly demonstrated that VKOR activity in liver arises chiefly from *VKORC1* expression by comparing VKOR activity levels from wild-type and *VKORC1*$^{-/-}$ mouse microsomes, which is in agreement with our mRNA data [6]. However, rescue experiments with daily vitamin K supplementation (30-50 μg/day) for *VKORC1*$^{-/-}$ mice resulted in life-saving, but physiologically low VKD

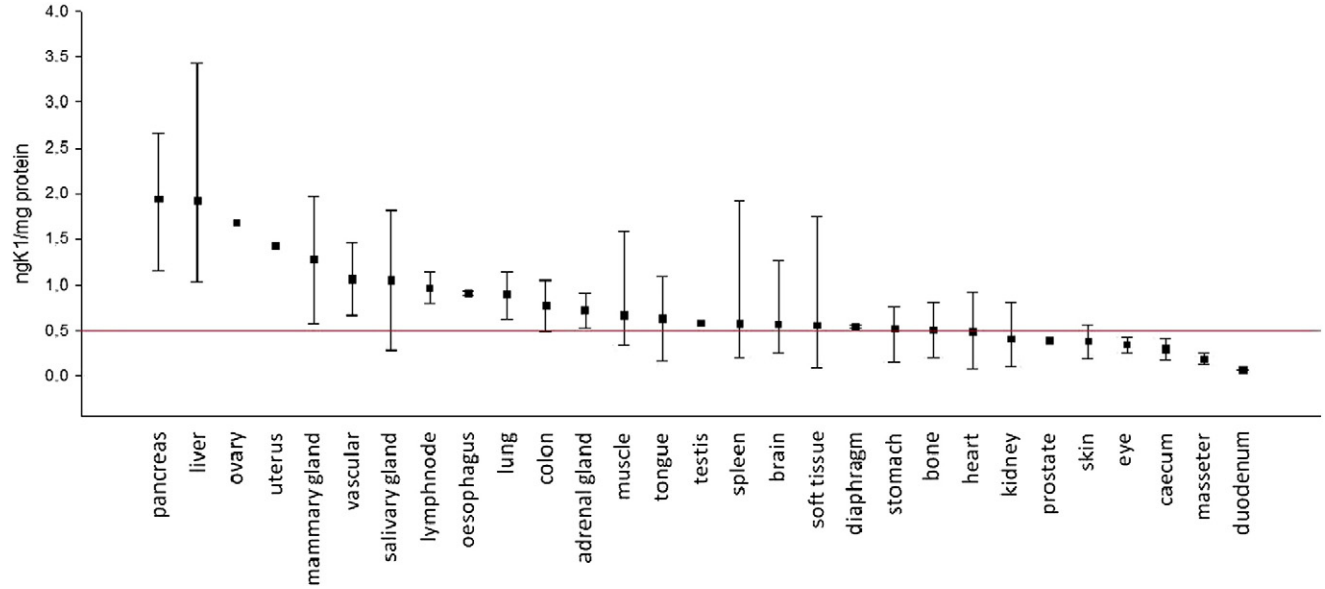

Fig. 3. VKOR activity measurements by the DTT-driven VKOR assay in 29 different mouse tissues. This figure show mean VKOR activities (ng K_1/mg total protein/h) for microsomes prepared from each tissue of 5 male and 5 female mice. Tissue types are indicated below the bars. Error bars show the ranges for highest and lowest measurements representing interindividual differences between mice. The red line indicate VKOR activity of 0.5 ng K_1/mg protein/h defined as basal VKOR activity.

coagulation factor activities [5]. Whether this residual activity can be attributed to low expression of *VKORC1L1* in liver detected in our study or, eventually to another still unknown vitamin K reductase enzyme, requires further investigation.

Furthermore, we found transcription of *NQO1*, a potential vitamin K reductase enzyme, to be low in liver - close to the detection limit. Highest mRNA expression of *NQO1* was found in gastrointestinal tissue (*e.g.*, stomach, caecum; Fig. 1D). Several *in vitro* studies have shown that NQO1 is able to reduce menadione (vitamin K_3 quinone) and is far less 4-hydroxycoumarin sensitive than VKORC1. Earlier, Wallin *et al.* suggested NQO1 might be the bypass enzyme for VKORC1 in cases of 4-hydroxycoumarin intoxication during high-dose application of vitamin K as an antidote [34]. However, a more recent *in vitro* study showed that NQO1 does not extensively catalyse vitamin K reduction during γ-carboxylation of VKD proteins [7]. Additionally, investigations using HEK293T and AV12 cells, as well as a recent study using *NQO1* knockout mice, revealed that NQO1 has no detectable function in the classical vitamin K cycle and, thus, is not the long-thought bypass enzyme [35, 11]. As *NQO1* transcription in liver is only low-level, our results provide further evidence that NQO1 does not contribute to the classical vitamin K cycle and, subsequently, to γ-carboxylation of VKD proteins. Also, in all other tissues investigated there is no positive correlation between the mRNA transcription levels of *VKORC1, GGCX* and *NQO1*.

Besides the high transcription levels of *VKORC1* and *GGCX* in liver, mRNA quantification revealed ubiquitous expression of *VKORC1* and *GGCX* in all 29 tissues investigated. Most interestingly, a high expression and VKOR activity in extrahepatic secretory tissues like salivary gland, lung, prostate, and mammary gland (for female mice) were found (Figs. 1A,B, and 4A).

It was reported that the VKD protein MGP is highly expressed in adult lung. MGP is known to act as calcification inhibitor and might be a candidate for this function in lung also [36]. $MGP^{-/-}$ mice die about eight weeks after birth due to aortic rupture. By that time, all elastic fibers in the vascular media of medium and large arterial vessels are completely calcified [36]. It was also reported that patients treated with 4-hydroxycoumarin oral anticoagulants, which directly inhibit

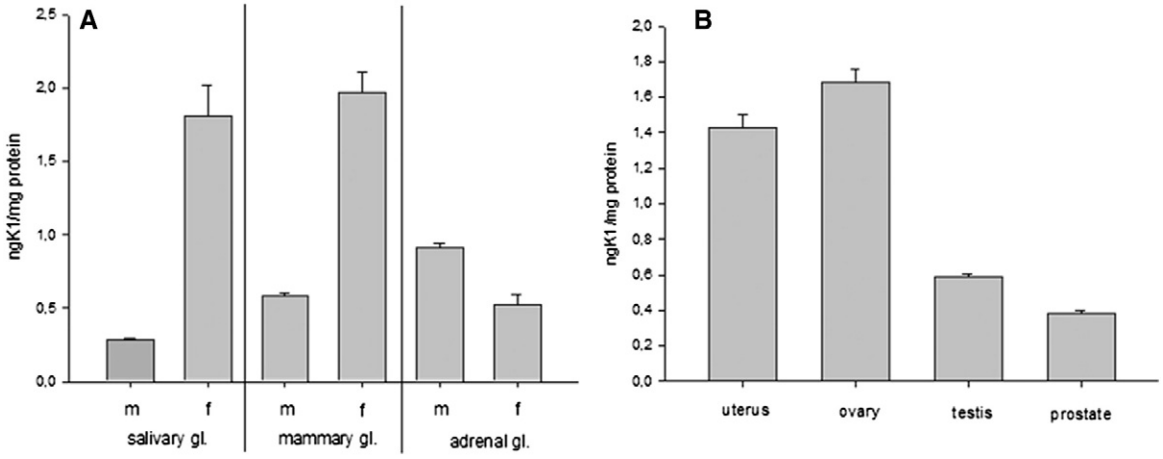

Fig. 4. Gender-specific VKOR activity in glandular tissue and sex organs. Panel A: Mean VKOR activity values for microsomes for salivary gland, mammary gland and adrenal gland (expressed in ng K_1/mg total protein/h) for female (labeled as "f") and male (labeled as "m"). Panel B: Mean VKOR activity for microsomes for uterus, ovary, testis and prostate. Error bars represent the SEM for each measurement (pooled samples of 5 male or 5 female mice).

VKORC1, showed two-fold greater calcification of aortic heart valves [37]. Therefore, MGP might be needed to protect the lung vessels against calcification - especially in the alveoli, where oxygen and carbon dioxide exchange takes place [36,38]. In addition, MGP is believed to be a key protein involved in the manifestation of warfarin embryopathy. Interestingly, MGP is not expressed during early lung development [36]. This absence of MGP expression possibly explains why no pathological lung phenotypes are observed in cases of warfarin embryopathy. Warfarin embryopathy is induced by warfarin administration during pregnancy and is asscociated with hypoplasia of the nasal bridge, stippled epiphyses and bone growth retardation. In this chondrodysplasia punctata-like phenotype, hyaline cartilage is hypercalcified especially in joints, larynx, and trachea. Hyaline cartilage of the epiphyses seems to be also affected in *VKORC1*$^{-/-}$ mice, which are born with shorter bones [5]. Additionally, in *MGP*$^{-/-}$ mice, vascular smooth muscle cells (VSMCs) differentiate to bone-like cells. Thus, other types of smooth muscle cells might also be affected by deficiency of the classical vitamin K cycle. Smooth muscle cells are not only found in walls of blood vessels or bronchioles of lung, but are also found in lymphatic vessels, urinary bladder, uterus, male and female reproductive tract, gastrointestinal tract, and respiratory tract. Therefore, MGP may have a protective function in other types of smooth muscle and might explain the additional high transcription of *VKORC1* and *GGCX* in prostate and mammary gland. Vena cava and aorta, which contain VSMCs, show fairly high expression of *VKORC1* and *GGCX*, consistent with MGP being protective against calcification.

The high transcription levels of *VKORC1* and *GGCX* we have detected in exocrine tissues including salivary gland, prostate, lung and mammary gland may be correlated and, thus, may point to roles for as yet unknown VKD proteins in exocrine tissues.

Addressing the function of VKORC1L1, Westhofen *et al.* first demonstrated the involvement of this enzyme in vitamin K-dependent intracellular antioxidation and proposed VKORC1L1 may be responsible for the transfer of reducing equivalents to ER membrane-associated antioxidative pathways [4]. In concordance with this hypothesis of universal cellular ROS protection, our data show a uniform basal expression of *VKORC1L1* in all tissues investigated (Fig. 1C). However, highest transcription of *VKORC1L1* was found in brain at 2.5-fold higher mean levels compared to salivary gland, the tissue with the next-highest *VKORC1L1* transcription level. Brain generates greater levels of free radicals than any other organ and is particulary susceptible to oxidative stress [39]. Therefore, oxidative cell-damaging free radicals and free radical-generating processes must be neutralized by antioxidative defence mechanisms. [40] Due to the substantial requirement for antioxidative activity in brain, we hypothesize that high transcription levels of *VKORC1L1* might be related to ROS scavenging and oxidative protection in brain (Fig. 1C).

In conclusion, our data demonstrate ubiquitous mRNA transcription for *VKORC1, VKORC1L1* and *GGCX*, as well as ubiquitous VKOR activity, for all tissues examined, suggesting an important role of the vitamin K cycle in extrahepatic as well as in hepatic tisues. A quantitative comparison regarding differential transcription levels revealed liver as the tissue of highest *VKORC1* and *GGCX* expression, and brain as the tissue of highest *VKORC1L1* expression. The very low transcription level of *VKORC1L1* in liver supports the exclusion of VKORC1L1 as a bypass enzyme for VKORC1. Furthermore, low transcription levels of *NQO1* in liver indicates that NQO1 has no function in the classical vitamin K cycle with respect to VKD coagulation proteins. High *VKORC1* and *GGCX* transcription levels toghether with VKOR activity levels in exocrine tissues including lung, female mammary glands, salivary gland, prostate and pancreas point to a substantial role of the classical vitamin K cycle in these tissues. γ-carboxylation of VKD proteins such as MGP appears to be essential for homeostasis of hyaline cartilage as well as of several types of smooth muscle cells. Further investigation is necessary to characterize the role of VKOR activity in extrahepatic tissues, to elucidate specific functions of VKD proteins and to identify new VKD post-translationally modified proteins.

Authorship Contributions and Conflict of Interest Disclosure

Experimental design (MC, KJC, JM, PW, MW, JO), data collection (MC, KJC, KL), data analysis (MC, KJC), manuscript drafting and editing (MC, KJC, MW, JO). The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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