Chapter 8

Anti-Inflammatory Actions of Vitamin K

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Abstract

Naphthoquinone compounds have received attention for their ability to regulate diseases from bacterial and parasite infections through to chronic human diseases. Inflammation is widely considered to be at the root of many chronic diseases. The reports of anti-inflammatory activity of naphthoquinones, including vitamin K1 (phylloquinone) and vitamin K2s (menaquinones), are of interest due to their very low toxicity. Most of the evidence for the anti-inflammatory mechanisms of vitamin K suggests a role in the inhibition of the cell signalling complex nuclear factor kappa-B (NF-κB).

Keywords: vitamin K1, vitamin K2, menaquinone, cell signalling, NF-κB

1. Introduction

From the 1960s through to the late 1990s, the F. Hoffman-La Roche laboratories in Basal, Switzerland, were the centre of much of the innovative investigation into the role of vitamins in physiology and pathophysiology. At that time, their laboratories were the leaders in exploring newer concepts of biological actions of vitamins. For vitamin K, an exciting potential development was in the analgesic and anti-inflammatory properties of naphthoquinone compounds; an area that had not previously been considered [1, 2].

The explosion in the understanding of several areas since then, such as cytokine and chemokine biology and physiology, molecular biology of signalling pathways and the genetic translation of these signals, has facilitated the ability to explore molecular events in the cell. With these innovations have come newer understandings of the role of vitamin K in physiology that go

beyond the gamma-carboxylation of specific protein glutamyl residues in the vitamin K–dependent proteins [3, 4].

2. Inflammation

The answer to the fundamental question 'What is Inflammation?' is, of course, complex. For many years, discussion on inflammation revolved around the immune system and the ability to distinguish 'self' from 'non-self'. This required an understanding of the ancient 'innate' immune system and the 'adaptive' immune system. There has been a sea change in thinking over the past three decades towards an appreciation of a primary role in the initiation of the inflammatory response residing in the tissues [5].

The cells of a tissue, or tissues, in an organism are primed to respond when they are exposed to an unusual stimulus. The response leads to a set of consequences, such as eicosanoid, cytokine, chemokine release, alterations in metabolic activity and genes becoming activated or switched off. These events can cause further cell activation either intrinsically within the tissue or extrinsically such as drawing leukocytes into the tissue. The whole process would be described as an inflammatory response.

The sequelae to the initiating events can lead to an expansion of the response, or the challenge can be nullified and the tissues return to an original state. If the inflammatory state continues, it can either persist as a long-term process, encountered as chronic diseases, or in severe cases, the explosive changes overwhelm the organism leading to tissue necrosis and organ failure [6, 7].

There are reasonable arguments to suggest that normal ageing shares some similarities with such chronic inflammatory disease [8, 9]. Indeed, the term 'inflammaging' was coined to describe this possibility [9]. This case has also been made for specific diseases of ageing, such as osteoporosis [10, 11].

There is also an innate anti-inflammatory process involving leukocytes and a number of biological mediators, such as cytokines; however, with respect to vitamin K, this area of research has not been the subject of focused research.

Irrespective of how we currently define inflammation, it is a biological process that serves a purpose in the preservation of the organism, sometimes at the cost of some part of the whole, but the government of the system can all too easily slip free of controls [12].

3. Vitamin K and inflammation

The pharmaceutical development of the anti-inflammatory role of activated protein C, a vitamin K–dependent protein, is being established at the boundary of inflammation and coagulation [13]. We will not consider this important subject here as, while vitamin K is required for generation of the mature functional protein, it is the role of protein C as a serine

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protease that is central to the mechanism of limiting microthrombus formation in organ tissue beds in sepsis [14].

Several important chronic diseases with an inflammatory background have been associated with vitamin K deficiency. These include cystic fibrosis, inflammatory bowel disease, pancreatitis, chronic kidney disease and osteoporosis [15–19]. This review will not address the relationship between vitamin K and these diseases, despite our ongoing interests. We have instead focused our attention on the proposed cellular and molecular aspects of vitamin K in regulating inflammation. With respect to this position, a subject that receives continuing interest is the potential role of vitamin K in the amelioration of signal transduction pathways; specifically, how vitamin K may be able to modulate the stimulus received at the surface of a cell and the message transmission to the cell nucleus for interpretation and response.

3.1. Introduction to cell-to-nucleus signalling

Within cells, there are several primary pathways that are important in communicating the exposure to an insult or injury. Early work on avian retroviruses, and in particular reticuloendotheliosis virus strain T (Rev-T), identified an oncogene (v-rel) capable of transforming avian lymphoid cells [20–22]. Once this oncogene had been characterized in avian systems, work progressed quickly to look at mammalian homologues. This research revealed a mouse homologue, c-rel, which was also found to have homologous character to the fruit fly Drosophila gene '*dorsal*' [23–25]. The Drosophila *dorsal* gene is known to play a fundamental role in dorsal-ventral development of the fly larvae, acting as a translocation factor in the cell nucleus to regulate gene expression. Around the same time, another nuclear translocation factor, with sequencing homology to these signalling factors, was discovered, which became known as nuclear factor kappa-B (NF-κB) [26].

3.1.1. NF-κB

We present a greatly simplified overview of NF-κB signalling, and we recommend that interested readers find some of the excellent review literature that has been published [e.g. 27–34] in a complex and engrossing story.

NF-κB is present in nearly all cells and participates in a diverse range of biological functions including inflammation, immunity, differentiation, cell growth, tumourigenesis and apoptosis, that is, from birth to death of a cell. This pathway is a central conductor of the molecular orchestra that is important for normal cell function, but it can become over-activated on a more general level [35] or to a greater degree leading to tumourigenesis [36, 37].

We now know that NF-κB is a family of proteins that exist as hetero- or homodimers that have been conserved from primitive organisms through to man [30]. The dimers are normally quiescent in the cytosol of cells through the close association with a regulatory inhibitory protein (IκB), first identified through an inspirational series of denaturation-renaturation experiments [38]. NF-κB becomes activated by the removal of IκB from the complex, which requires IκB phosphorylation by cytosolic kinase enzymes (IKK) [39]. The IκB protein is then tagged, by ubiquitination, for degradation in the proteasome [34, 40]. A family of IκBs have

now been identified and together with the IKK enzymes that phosphorylate the IκB proteins and a scaffold IKK modulator (NEMO; IKKγ) [41], function at the heart of the system of NF-κB activation and regulation.

Once dissociated from its inhibitory chaperone, NF-κB moves to the cell nucleus where it recognizes specific nucleotide promoter sequences that activates a number of genes. This is known for over 200 genes.

The complexity of the activation of NF-κB creates multiple tiers of regulatory control and, therefore, also potential focal points for the development of therapeutic agents. Vitamin K and its derivatives have been investigated for their ability to intervene in the activation of NF-κB.

3.1.2. NF-κB and vitamin K

There is evidence that vitamin K can regulate the activation of the NF-κB pathway. Other signalling pathways also control cell functions and are, of course, important. Furthermore, in the complex nature of multiple signalling pathways there is also considerable crosstalk between these pathways [42]. There are also some reports that vitamin K has regulatory functions on signalling pathways, other than NF-κB, such as the mitogen-activated protein (MAP) kinases, but in this discussion, we focus on vitamin K and NF-κB.

Most of the research on vitamin K regulation of the NF-κB pathway has been done on cultured cells, with a large element coming from researchers in Japan with a core interest in the molecular mechanisms regulating cancer cells.

Hepatocellular carcinoma cells (HCC) are known to overexpress several oncogenes and also down-regulate tumour suppressor genes [43, 44]. Vitamin K2, in the form of menaquinone-4, has been investigated for its growth regulating effects in three hepatocellular cancer cell lines [45]. Focussing on the proto-oncogenic expressed protein cyclin D1, which regulates the cell cycle at the G_1-S transition, and which is itself regulated by NF-κB [46]. These researchers found menaquinione-4 inhibited both cyclin D1 mRNA expression and protein synthesis. In HCC activated with phorbol ester or cytokines agonists in order to stimulate NF-κB signalling, vitamin K was found to ameliorate cyclin D1 activation and NF-κB promoter binding was inhibited. It was also noted that there was inhibition of the phosphorylation of IκB, with suppression of IKK kinase activity.

The same research group has considered the effects of vitamin K2 on the expression of matrix metalloproteinase (MMP) enzymes [47]. These enzymes are implicated in metastatic tumour invasion [48]. HCC in culture were treated with phorbol ester and the expression of three MMP proteins and their corresponding mRNA examined. The study found that menaquinone-4 suppressed basal and phorbol ester stimulated NF-κB activation, which translated into the suppression of both MMP mRNA and protein expression levels. In these experiments, vitamin K2 was also found to suppress the activation of the mitogen-activated protein (MAP) kinase signalling pathway.

Two other publications from these researchers, again using HCC, explored the effects of vitamin K2 to regulate cancer cell biology [49, 50]. In the first they examined potential

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modulation of HCC growth by the anti-cancer therapeutic 5-fluorouracil, through inhibition of cyclin D and innate activated NF-κB signalling. Their other study investigated the effects of vitamin K2 on protein kinase C (PKC), a known mediator in multiple stimulated cell responses in HCC [51]. Working in an adjunctive role with 5-fluorouracil, menaquinone-4 was found to augment the growth inhibition induced by 5-fluorouracil alone, this being most effective when the cells were pretreated with vitamin K2. From the discussion above on the NF-κB pathway, any finding of an inhibition of kinase activities may be expected to have an effect on NF-κB activation. In their PKC study, these researchers found delineation in the response to vitamin K2 between the PKC isoforms. All the PKC isozymes were found to be involved in NF-κB activation, but vitamin K2 inhibited the NF-κB activation through its actions on only two of these, PKCα and PKCε. This selectivity was not investigated further.

The above discussion focuses on directed inflammatory stimuli using laboratory reagents; however, there are numerous agents that can promote an inflammatory response, including infective agents like viruses and bacteria. We are all exposed to bacterial endotoxins or lipopolysaccharides (LPS), every day and without much effect, but when these endotoxins are shed in an injured tissue, the results are registered quickly through cell surface Toll-like receptors that trigger, among other signalling pathways, NF-κB [52].

A potent, and key, cytokine in the response to endotoxin insult is interleukin-6 (IL-6) [53]. Up-regulation of IL-6 synthesis is under NF-κB control [54], and this cytokine can feed back into the inflammatory response. This feedback is achieved via interaction with its own specific cell surface receptor that activates the Janus kinase (JAK) signal transducer and activator of transcription 3 (STAT3) pathway [55].

We have reported a vitamin K-mediated suppression of IL-6 release in LPS-challenged primary human fibroblast cell cultures [56]. This investigation found that vitamin K2, in the form of menaquinone-4, was a more potent anti-inflammatory compound than vitamin K1 in this primary cell system.

Other cell and animal experiments have looked at the possible regulation of inflammation by vitamin K inhibition of IL-6 release following endotoxin challenge. These include studies using germ-free rats, THP-1 human monocyte-like cells and murine RAW264.7 macrophage-like cells [57, 58] and have noted a significant reduction in the LPS-stimulated cellular IL-6 mRNA levels. In the vitamin K supplemented animal experiments, there was a suppression of the hepatic mRNA levels of the inflammation response protein macrophage migration inhibitory factor (MIF), compared to animals fed a vitamin K-deficient diet. This study also found that supplementation with vitamin K1 also caused a rise in hepatic tissue menaquinone-4 levels [57]. Extension of these *in vivo* findings in the two cell lines, THP-1 and RAW264.7, demonstrated that the suppression of LPS-stimulated IL-6 mRNA by vitamin K2 was consistent with inhibition of NF-κB activity via the restricted phosphorylation of IKK kinases [58]. These researchers also questioned whether these events involved a classical vitamin K gamma-carboxylation-mediated process; co-culture experiments with warfarin found that these inhibitory effects of vitamin K on NF-κB were independent of vitamin K–dependent protein gamma-carboxylation [58]. The widely appreciated role of IL-6 in inflammatory diseases

makes the findings of an inhibitory activity for vitamin K in IL-6 release an increasingly attractive prospect.

One of the dominant areas of NF-κB understanding is in osteoclastogenesis. Activation of this pathway in hematopoietic stem cells by receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the tumour necrosis ligand superfamily, has been found to be a prominent initiator of osteoclast formation [59, 60]. Once precursor cells have been positioned to develop into osteoclasts by RANKL, NF-κB further facilitates development through inhibition of apoptosis [61].

Menaquinone-7, a more hydrophobic homologue of vitamin K2 than menaquinone-4, has been found to limit rat osteoclast formation under various challenges [62]. Later studies determined that the anti-osteoclastogenic actions of menaquinone-7 were mediated by the inhibition of NF-κB [63].

A central feature of all research on the role of vitamin K in the inhibition of NF-κB is the high concentrations that were required to demonstrate an effect of the order of 10^{-5} to 10^{-4} M in cell culture and pharmacological dosing in animal experiments. The relatively high concentrations required suggests that the anti-inflammatory activities of vitamin K are not working through specific receptors and it is, therefore, more likely that the anti-inflammatory activities are due to other characteristics of vitamin K.

3.2. Other anti-inflammatory vitamin K–like compounds

Some supporting data on the potential for vitamin K to serve a physiological anti-inflammatory role can be found in studies using other vitamin K–like compounds. Below, we focus on two compounds that have a common 2-methyl-1,4-naphthoquinone functional group as vitamin

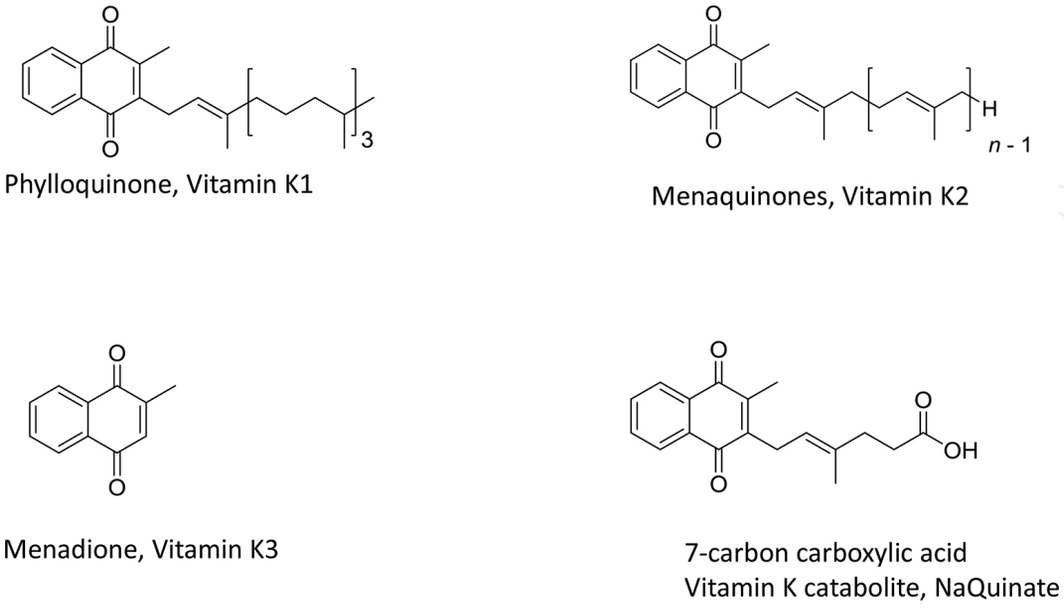

Figure 1. The molecular structures for phylloquinone, vitamin K1; menaquinone vitamins, vitamins K2; menadione, vitamin K3; NaQuinate, the 7-carbon carboxylic acid catabolite derived from all forms of vitamin K1 and K2.

K1 and K2, namely menadione, or vitamin K3, and a 7-carbon carboxylic acid catabolite of vitamin K, NaQuinate (**Figure 1**).

3.2.1. Menadione and inhibition of inflammation

Menadione, or vitamin K3, was for a long time considered to be a synthetic compound. Research that started in the 1990s demonstrated that menadione was an important derivative from vitamin K1 that was converted in the rat to make the vitamin K2 congener menaquinone-4 [64–67]. The enzyme responsible for the conversion of menadione to menaquinone-4, UBIAD1 [68] has a wide distribution across species and likely plays a significant role in the vitamin K status of many animals (see chapter by O'Neil et al., this volume).

Seminal work on the amelioration of chronic inflammatory arthritis in a rabbit antigen-induced model found a pronounced anti-inflammatory effect in animals receiving oral menadione. This was related to a significant decrease in synoviocyte glucose-6-phosphate dehydrogenase activity in the treated rabbits [69] and reduced macrophage numbers in the treated joint (personal communication). It is unclear if this was due to a direct effect on the joint tissue as a latter study using menadione-epoxide in the same model was also found to have a significant anti-inflammatory activity [70]. Alternative explanation for these effects includes the possible conversion of these menadione and menadione-epoxide molecules into vitamin K2, as menaquinone-4, through the action of UBIAD1 [68]. This would be consistent with lapine caecal bacterial conversion due to known coprophagy in this species (see chapter by O'Neil et al., in this volume).

Menadione has also been shown to inhibit NF-κB translocation to the nucleus in tumour necrosis factor (TNF)-α stimulated human embryonic kidney (HEK)293 cells [71]. Intriguingly, such effects were not observed in similar experiments using vitamin K1 or K2 in these cells. There are several possible reasons for this divergence in activity between the naphthoquinone homologues. However, menadione is known to be inert as a co-factor for the vitamin K–dependent gamma-carboxylation reaction [72], suggesting that alternative mechanisms, related to their redox properties, rather than protein modification are likely to be more dominant.

These researchers also found that menadione was capable of suppressing LPS-induced NF-κB nuclear translocation and TNF-α release from murine macrophage-like RAW 264 cells [71]. Additionally, in a murine model of acute lung injury/acute respiratory distress syndrome (ARDS), which occurs in the setting of acute severe illness complicated by systemic inflammation, menadione also attenuated the LPS-induced severity of lung injury and suppressed the increase in serum TNF-α level. This occurred concomitantly with inhibition the LPS-evoked nuclear translocation of NF-κB in lung tissue.

Together, these data indicate that menadione has a capacity to ameliorate inflammation. The *in vivo* examples identified above can be described as rigorous tests of the anti-inflammatory potential of this molecule. The mechanism of action is likely to centre on NF-κB, but the strong suppression of glucose-6-phosphate dehydrogenase in the rabbit arthritis model and, thereby,

the regulation of intracellular NADPH, may indicate another important feature of the anti-inflammatory character of menadione.

3.2.2. Inhibition of inflammation by a vitamin K catabolite, NaQuinate

Vitamin K1 and K2s are broken down in the liver, and this is the only organ that has been reported to be responsible for the catabolism of these vitamins [73]. Early metabolism studies in men using radioactive vitamin K1 has revealed the catabolites to be side-chain shortened carboxylic acid products, the more abundant being a 5-carbon aliphatic acid, while the less abundant is a 7-carbon aliphatic acid containing a single double bond (**Figure 2**) [74]. These compounds are glucuronidated in the liver for excretion in the bile or through the kidneys. In human studies, the level of the catabolites varies with the amount of vitamin K ingested at pharmacological doses, as either phylloquinone or menaquinone-4 [75] or with dietary phylloquinone intake [76].

We have found that both of these acid catabolites cannot participate in the gamma-carboxylation reaction and are, in fact, inhibitors or the vitamin K gamma-carboxylase enzyme (Soper and Hodges, unpublished data).

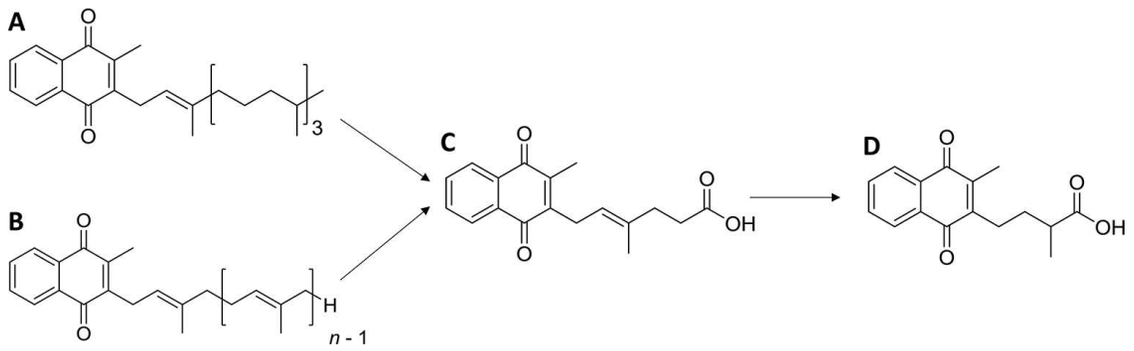

Figure 2. The metabolism of vitamin K1 (A) and vitamins K2 (B) with the common generation is the 7-carbon carboxylic acid catabolite, NaQuinate (C), which is further broken down to the 5-carbon carboxylic acid catabolite (D).

Our recent studies with the 7-carbon carboxylic acid catabolite of vitamin K, NaQuinate (**Figure 1**), found significant attenuation of LPS-challenged osteoblast-like MG63 cell IL-6 release [77]. This has likely *in vivo* implications, as in humans NaQuinate is usually present at high levels following pharmacological vitamin K dosing, irrespective of the source of vitamin K as either vitamin K1 or any of the vitamin K2 vitamers (**Figure 2**) [75]. Parallel experiments in MG63 cells using 1,25-$(OH)_2$-vitamin D3 or interleukin-1β as agonists showed similar suppression of IL-6 release by NaQuinate (Soper and Hodges, unpublished data). Interestingly, LPS-challenged MG63 cells were substantially less affected by the 5-carbon carboxylic acid vitamin K catabolite (**Figure 2**), which only differs from NaQuinate by 2 carbon atoms and a carbon-carbon double bond [77].

These results are in agreement with our earlier findings with LPS-challenged primary human fibroblast cultures [56]. Furthermore, if the carboxylic acid function on the catabolites is blocked with a methyl group, the LPS-induced release of IL-6 is greatly reduced in MG63 cells

[77]. The reason for the disparity in inhibitory activities between the two closely related vitamin K catabolites is not known, and a free acid function is a common structural element of many widely used anti-inflammatory agents.

It should be noted that, in comparison to the descriptions above of the effects of vitamin K on NF-κB and other anti-inflammatory markers, the concentration of NaQuinate required to achieve an effect was substantially lower, with an IC_{50} of $2–5 \times 10^{-7}$ M.

More recently, *in vivo* investigations have been performed using NaQuinate in two murine models of bone loss, induced through either ovariectomy (OVx), or as a result of limb disuse following sciatic neurectomy (NTx). In the OVx model, we found, using several micro-computed tomography measurement parameters, that tibial trabecular bone loss was significantly ameliorated in NaQuinate treated animals (0.75 mg/kg); the tibiae in the sham-operated control group being indistinguishable from the NaQuinate-treated oviarectomised animals [77]. We found that the more aggressive bone loss following NTx was also greatly reduced in the NaQuinate-treated animals. Moreover, we found substantial reduction in the inhibition of bone loss in both of the mouse models when a NaQuinate derivative containing a methyl ester group was used to block the carboxylic acid function, suggesting that a free carboxylic acid function is required for effective inhibition of bone loss in these models.

4. Concluding remarks

Vitamin K has been found to have anti-inflammatory activity in an increasing number of studies. This inhibitory activity would appear to be directed through inhibition of NF-κB signalling. In cell culture experiments, it is often mentioned that the cells needed to be primed with vitamin K before being challenged with agonists. Furthermore, experimental results are immerging that show inhibition of cytokine release in agonist-challenged cell culture and animal models, which in the light of vitamin K deficiency in chronic diseases, may reflect on a vitamin K role in organ homeostasis. Therefore, the defining vitamin K sufficiency on the basis solely of adequate functional blood clotting factors is likely to be an over-simplification and needs to be considered more fully.

The question is open as to the value of vitamin K in a therapeutic or a prophylactic role. For example, a vitamin K deficient, seriously ill patient is unlikely to benefit greatly from a vitamin K intervention, but a patient with a chronic inflammatory disease may. This can only be highlighted in large community studies, which need to be run over many years in large numbers of volunteers.

However, there are already some indications of prophylactic benefit of increased vitamin K intake in large population studies in Japan. Exploring consumption of 'natto', a live menaquinone-7 producing bacteria (*Bacillus subtilis* subsp) culture food product from fermented soybean that is culturally more favoured in the east than the west of Japan found that ingestion substantially increases human circulating menaquinone-7 levels and that bacterial gut colonization was evident several days after a single 80 g natto portion [78]. Intriguingly,

prefectural sales of natto also linked consumption with a notable decrease in age-related hip fractures in eastern Japanese population [78].

Given the safety of vitamin K, even in high pharmaceutical doses, supplementation may have a benefit, beyond meeting coagulation demands than is generally perceived, particularly in early chronic inflammatory diseases and inflammaging.

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