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**Vitamin K2 Covalently Binds to Bak and Induces Bak-Mediated Apoptosis**

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Bak as a molecular target of vitamin K2-induced apoptosis

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### **Abbreviations:**

VK, vitamin K; Bak, Bcl-2 antagonist killer 1; MK, menaquinone; GGCX,  $\gamma$ -glutamyl carboxylase; SXR, steroid and xenobiotic receptor; HSD17B4, 17 $\beta$ -hydroxysteroid dehydrogenase 4; MDS, myelodysplastic syndrome; PARP, poly (ADP-ribose) polymerase; CHX, cycloheximide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FCCP, carbonyl cyanide

4-(trifluoromethoxy)phenylhydrazone; APF, aminophenyl fluorescein; NHS,  
N-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide  
hydrochloride; FD5M, fluorescein diacetate 5-maleimide; Cyt c, cytochrome c; VK2-O, VK2  
2,3-epoxide; VK2-H2, VK2 hydroquinone; ROS, reactive oxygen species; VKORC1L1,  
vitamin K epoxide reductase complex, subunit 1-like 1; VKORC1, vitamin K epoxide  
reductase complex, subunit 1.

## ABSTRACT

Vitamin K2 (VK2, menaquinone) is known to have anticancer activity *in vitro* and *in vivo*. Although its effect is thought to be mediated, at least in part, by the induction of apoptosis, the underlying molecular mechanism remains elusive. Here, we identified Bcl-2 antagonist killer 1 (Bak) as a molecular target of VK2-induced apoptosis. VK2 directly interacts with Bak and induces mitochondrial-mediated apoptosis. Although Bak and Bax, another member of the Bcl-2 family, are generally thought to be functionally redundant, only Bak is necessary and sufficient for VK2-induced cytochrome c release and cell death. Moreover, VK2-2,3 epoxide, an intracellular metabolite of VK2, was shown to covalently bind to the cysteine-166 residue of Bak. Several lines of evidence suggested that the covalent attachment of VK2 is critical for apoptosis induction. Thus, this study reveals a specific role for Bak in mitochondria-mediated apoptosis. This study also provides insight into the anticancer effects of VK2 and suggests that Bak may be a potential target of cancer therapy.

## INTRODUCTION

Vitamin K (VK) is a fat-soluble vitamin that was discovered in 1929 (Dam, 1929). There are three types of VK: naturally occurring VK1 (phylloquinone) and VK2 (menaquinone, MK), and chemically synthesized VK3 (menadione). VK2 is also known as MK-n (n=1 to 14), where n stands for the number of repeating isoprenyl units in its side chain (Supplementary Fig. 1). The most common form of VK in animals is MK-4, which is produced by intestinal bacteria or is metabolically converted from other VKs (Seegers and Bang, 1967). VK was originally discovered as an essential factor for blood coagulation (Dam, 1935). VK acts as a cofactor for  $\gamma$ -glutamyl carboxylase (GGCX), which catalyzes the carboxylation of specific glutamic acid residues ( $\gamma$ -carboxylation) of substrate proteins. The substrates of VK include blood coagulation factors such as factor VII, factor IX, and factor X, and the  $\gamma$ -carboxylation of these factors is essential for their function (Stafford, 2005). Moreover, VK-dependent  $\gamma$ -carboxylation plays an important role in bone homeostasis. Osteocalcin, a critical regulator of calcium uptake and bone mineralization in osteoblasts, is activated by  $\gamma$ -carboxylation (Hauschka et al., 1975). Vitamin K deficiency causes bleeding diathesis, particularly in newborn babies (Thorp et al., 1995). In addition, undercarboxylation of osteocalcin due to vitamin K deficiency is thought to result in osteoporosis (Iwamoto et al., 2004). Thus, MK-4, one of the most potent VKs, has been widely used as a therapeutic drug for the above-mentioned diseases (Ushiroyama et al., 2002).

In addition to its role as a cofactor of GGCX, VK2 has been reported to play other roles by binding to different intracellular proteins. A chemical genetic screen revealed that VK2 binds and activates steroid and xenobiotic receptor (SXR), an orphan receptor of the nuclear receptor superfamily, thereby inducing the transcription of extracellular matrix-related genes in the human osteoblast cell line MG-63 (Tabb et al., 2003; Ichikawa et al., 2006). In another study, 17 $\beta$ -hydroxysteroid dehydrogenase 4 (HSD17B4) was identified as a VK2-binding protein by affinity purification using biotinylated VK2. HSD17B4 catalyzes the conversion of estradiol (E2) to estrone (E1), and VK2 was found to decrease the E2:E1 ratio in the human hepatoma cell line HepG2 (Otsuka et al., 2005).

Apart from these activities, VK2 also exerts anticancer activity *in vitro* and *in vivo*. Previous studies have demonstrated that VK2 inhibits proliferation of various cancer cells, such as leukemia and hepatocellular carcinoma (Matsumoto et al., 2006; Yokoyama et al., 2008). Clinical studies have reported that VK2 is effective in the treatment of myelodysplastic syndrome (MDS) and post-MDS acute myeloid leukemia (Yaguchi et al., 1999; Miyazawa et al., 2000). Moreover, VK2 prevents the development or recurrence of hepatocellular carcinoma in patients with viral cirrhosis (Habu et al., 2004; Mizuta et al., 2006). VK2 is not used in clinical practice because its anticancer activity is not sufficiently strong; however, the lack of serious adverse effects associated with VK2 treatment, even at high doses, makes it a potentially useful alternative to currently available anticancer drugs.

Compared to its effects on blood coagulation and bone homeostasis, the mechanisms underlying the anticancer effects of VK2 are not well understood. Previous studies have suggested the involvement of apoptosis induction, cell-cycle arrest, and differentiation (Tokita et al., 2005; Tsujioka et al., 2006). Furthermore, studies have reported contradictory findings regarding the effect of VK2 in cancer cells. VK2 was reported to induce caspase-dependent apoptosis in human myeloma cells (Tsujioka et al., 2006). However, another study reported that VK2 induced apoptosis in a caspase-independent manner in human liver cancer cells (Matsumoto et al., 2006). The identification of a direct binding target of VK2 is important to elucidate the apparently complex roles of this molecule. As mentioned above, SXR and HSD17B4 have been identified as molecular targets of VK2. However, it is unlikely that these targets account for the molecular mechanisms of VK2-induced apoptosis, because to our knowledge, SXR does not control genes involved in apoptosis, and HSD17B4 is also unrelated to apoptosis. Hence, there is likely to be an unknown target(s) of VK2 that mediates its apoptosis-inducing effects. Because VK2 treatment is not associated with adverse effects, the identification of its target protein(s) would be helpful for the development of novel anticancer drugs with fewer side effects.

In this study, high-performance affinity beads, previously shown to be useful for the identification of drug target proteins (Shimizu et al., 2000; Ito et al., 2010), were used to

identify Bcl-2 antagonist killer 1 (Bak), a proapoptotic protein involved in mitochondrial-mediated apoptosis, as a molecular target of VK2-induced apoptosis.

## MATERIALS AND METHODS

**Antibodies and Chemicals.** Antibodies against actin (C4, Millipore, Billerica, MA), Bak (NT, Millipore), Bax (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), PARP-1/2 (H-250, Santa Cruz), Caspase-3 (Cell Signaling Technologies, Beverly, MA), cytochrome c (cyt c) (7H8.2C12, BD Pharmingen, San Diego, CA), GAPDH (Merck, Whitehouse Station, NJ), Bcl-2 (N-19, Santa Cruz), Bcl-xL (54H6, Cell Signaling Technologies), PHB1 (H-80, Santa Cruz), PHB2 (Millipore), VDAC1 (31HL, Millipore), and FLAG (M2, Sigma-Aldrich, St. Louis, MO) were purchased from commercial sources. VK2 (MK-4) was purchased from Eisai (Tokyo, Japan). Cycloheximide (CHX) (Sigma-Aldrich), z-VAD-fmk (Peptide Institute, Osaka, Japan), and alpha-tocopherol (Sigma-Aldrich) were obtained from commercial sources.

**Cell Culture.** The human promyelocytic cell line HL60 was cultured in Roswell Park Memorial Institute medium containing 10% fetal bovine serum (FBS). The human cervical carcinoma cell line HeLa was cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

Cell proliferation was examined by a modified MTT assay with SF reagent (Nacalai Tesque, Kyoto, Japan). Specifically, an aliquot of cell suspension was transferred to 96-well plates, and 10  $\mu$ l of SF reagent was added to each well. The absorbance at 450 nm was measured using a Wallac 1420 Arvo SX Multilabel Counter (Perkin Elmer, Waltham, MA). The mean absorbance value of the control group was arbitrarily set to 100%.

To prepare cytoplasmic extracts of HL60 cells for affinity purification, the cells were suspended in buffer A [10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. After incubation on ice for 15 min, CHAPS was added to a final concentration of 1%, and the lysate was centrifuged at 13,000  $\times$  g for 5 min. The supernatants were collected and dialyzed with buffer D (20 mM Hepes, pH 7.4, 100 mM KCl, and 1 mM EDTA). The dialyzed supernatants were used as cytoplasmic extracts. For the analysis of cyt c release, cytoplasmic extracts were



prepared as described previously (Matsura et al., 2002).

**Plasmid Construction.** The cDNAs encoding Bak and Bax were obtained from Dr. Shigeomi Shimizu (Tokyo Medical and Dental University, Tokyo, Japan). DNA fragments encoding HA-FLAG- or FLAG-tagged Bak and Bax were prepared by polymerase chain reactions and were inserted into pcDNA3.1 (+) (Life Technologies, Carlsbad, CA).

**Measurement of Mitochondrial Membrane Potential and ROS Generation.** In Fig. 1C, HL60 cells were incubated with 10 or 30  $\mu$ M VK2 for 48 h, or with 5  $\mu$ g/mL carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich) for 4 h. For the determination of mitochondrial membrane potential, cells were then treated with 10  $\mu$ M Rhodamine 123 (Wako Pure Chemical, Osaka, Japan) for 15 min at 37°C. In Fig. 2A, mitochondria were isolated from rat liver, as described previously (Hase et al., 2008). The isolated mitochondria were incubated with 10  $\mu$ M VK2 or 0.1  $\mu$ g/mL FCCP for 3 h and were then treated with 1  $\mu$ g/mL JC-1 (Wako Pure Chemical) for 1 h at 30°C. In Fig. 4A, HL60 cells were incubated under the indicated conditions for 24 h and then treated with aminophenyl fluorescein (APF) (Sekisui Medical, Tokyo, Japan) for 30 min at 37°C. Each sample was subjected to fluorescence-activated cell sorting analysis (BD Bioscience, San Jose, CA).

**Immunofluorescence Analysis of Bak and Bax Activation.** HeLa cells were seeded on cover slips and treated with 30  $\mu$ M VK2 for 48 h. Cells were then fixed with 1% paraformaldehyde for 15 min and permeabilized with PBS containing 0.2% Triton X-100. Active forms of Bak and Bax were visualized by using anti-Bak (Ab-1, Millipore) and anti-Bax (6A7, Medical & Biological Laboratories, Nagoya, Japan) antibodies, respectively, and secondary antibody conjugated with Alexa Fluor 488 (Life Technologies), followed by counterstaining with 4',6-diamidino-2-phenylindole. Fluorescent images were captured with an Olympus IX 81 microscope and analyzed using the MetaMorph software (Universal

Imaging, Downingtown, PA).

**Affinity Purification Using VK2-immobilized Beads.** A diagram of the immobilization procedure is shown in Supplementary Fig. 1. Carboxylated FG beads were incubated with 200 mM N-hydroxysuccinimide (NHS) and 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for 2 h and then incubated with 0.5 mM amino derivative of VK2 for 70 min at room temperature. Unreacted groups were blocked using 1 M ethanolamine. For the immobilization of VK2, we used an amino derivative of MK-2, in which the side chain containing an ethylene glycol is attached as a linker. A previous study showed that MK-2 possesses apoptosis-inducing activity (Suhara et al., 2008). For affinity purification, VK2-immobilized beads (0.2 mg) were incubated with 200  $\mu$ L (1 mg protein/mL) of HL60 cytoplasmic extract in buffer D containing 1% CHAPS at 4°C for 4 h. After the beads were washed three times, bound protein was eluted with SDS-PAGE sample buffer.

**Transient Transfection.** HeLa cells were transfected with pcDNA3.1 (+)-FLAG-Bak by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Four hours later, cells were replated in fresh medium with 10% FBS and 10  $\mu$ M zVAD-fmk.

For knockdown experiments, the following Stealth RNAi oligonucleotides (Life Technologies) were used: Bak, 5'-UGAAGAAUCUUCGUACCACAAACUG-3'; and Bax, 5'-ACUUUGCCAGCAAACUGGUGCUCAA-3'. Only sense strands are shown. Stealth RNAi negative control of low GC content (Life Technologies) was also used. HeLa cells were transfected with each Stealth oligonucleotide (20 nM) using Lipofectamine RNAiMAX (Life Technologies).

**Mass Spectrometry.** HeLa cells expressing FLAG-Bak were incubated with or without 30  $\mu$ M VK2 for 48 h. Cell lysates were then prepared and incubated with anti-FLAG

agarose beads (Sigma-Aldrich), and bound materials were eluted with FLAG peptide (Sigma-Aldrich). Purified FLAG-Bak in 0.1% trifluoroacetic acid (Wako Pure Chemical) was loaded at the flow rate of 300 nL/min onto a nano-HPLC capillary column (NTCC-360/75-3-110, 0.075 mm i.d. × 110 mm, Nikkyo Technos, Tokyo, Japan) installed in an Easy-nLC II liquid chromatography system (Thermo Scientific, Waltham, MA). For eluents A and B, 0.1% formic acid (LC/MS grade, Wako Pure Chemical) in distilled water (LC/MS grade, Wako Pure Chemical) and 0.1% formic acid in acetonitrile (LC/MS grade, Merck) were used, respectively. A linear gradient of eluent B from 0% to 30% in 2 min, and then from 30% to 70% in 20 min was used for elution. The nano-HPLC column was attached to the nano electrospray ion source of the LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. The average mass of each peak was calculated from mass spectra using the ProMass deconvolution software (Novatia, LLC, Monmouth Junction, NJ).

**Fluorescence Assay.** Reduced cysteine residues were labeled as described previously (Nishi et al., 2002). In brief, proteins were incubated with 0.3 mM fluorescein diacetate 5-maleimide (FD5M) (Sigma-Aldrich) for 5 min. The labeling reaction was stopped by the addition of 100 mM DTT. Proteins were acetone-precipitated, suspended in SDS-PAGE sample buffer, and then separated by SDS-PAGE. Fluorescence intensity of labeled proteins was determined by scanning gels at an excitation wavelength of 488 nm using the fluorescence-image analyzer Typhoon Trio (GE Healthcare, Little Chalfont, UK).

## RESULTS

**Vitamin K2 Induces Mitochondrial-Mediated Apoptosis in HL60 Cells.** The induction of apoptosis by VK2 was examined in the human promyelocytic cell line HL60 based on its sensitivity to VK2-induced apoptosis (Yokoyama et al., 2008). HL60 cells were treated with various concentrations of VK2 for 48 h, and cell viability was assessed by a soluble tetrazolium/formazan assay. As previously described (Yokoyama et al., 2008), VK2 inhibited cell proliferation in a concentration-dependent manner (Fig. 1A). Based on pharmacokinetic and pharmacodynamic studies in humans and animals (Ishii et al., 1992; Sano et al., 1995; Sano et al., 1997), the tissue concentration of VK2 is estimated to reach 10  $\mu$ M in patients who receive clinically relevant doses of VK2. In addition, the conversion of procaspase-3 to caspase-3, and the cleavage of poly (ADP-ribose) polymerase (PARP), one of the substrates of caspase-3, were observed after 48 h VK2 treatment, but not after 24 h treatment (Fig. 1B).

Because mitochondrial dysfunction is implicated in VK2-induced apoptosis (Shibayama-Imazu et al., 2008), we next examined the effect of VK2 on the mitochondrial-mediated apoptosis pathway. To examine the effect of VK2 on mitochondrial membrane potential, HL60 cells were treated with VK2 or the proton ionophore FCCP, and mitochondrial membrane potential was measured by flow cytometry using Rhodamine 123. As shown in Fig. 1C, VK2 disrupted mitochondrial membrane potential as well as FCCP. Assessment of the release of cyt c from mitochondria showed a VK2 concentration-dependent release of cyt c from mitochondria after 48 h (Fig. 1D). Because the loss of mitochondrial membrane potential and cyt c release are mediated by Bak and Bax (Wei et al., 2000; Korsmeyer et al., 2000), we examined these two proapoptotic Bcl-2 family proteins in human cervical carcinoma HeLa cells by immunofluorescence microscopy. After VK2 treatment, spotted fluorescent signals were detected using antibodies against the active forms of Bak and Bax (Fig. 1E), indicating that VK2 induces their activation and oligomerization. These results suggested that the effect of VK2 on the mitochondrial-mediated apoptosis pathway may be mediated by the activation of Bak and

Bax.

**Bak Interacts with VK2 and Mediates VK2-Induced Apoptosis.** To examine the mechanism of action of VK2, we investigated its potential effect on mitochondrial membrane properties. Mitochondria isolated from rat liver were treated with VK2 or FCCP, and the membrane potential was measured by flow cytometry using JC-1 staining. VK2 (10  $\mu$ M) disrupted the membrane potential of isolated mitochondria, although to a lesser extent than FCCP (Fig. 2A), suggesting that VK2 may exert its proapoptotic activity directly through a mitochondrial protein.

The above result led to the investigation of the possible effects of VK2 on mitochondrial proteins such as Bak, Bcl-2, and VDAC1. As shown in Fig. 2B, VK2 did not appreciably affect the expression levels of the proteins examined, but a slower-migrating band crossreacting with the anti-Bak antibody was detected after 48 h VK2 treatment. Moreover, a similar observation was made when HeLa cells transiently overexpressing FLAG-Bak were analyzed using anti-FLAG antibody (Fig. 2C). Here, following transient transfection of FLAG-Bak, the pan-caspase inhibitor zVAD-fmk was added to cell culture to prevent substantial cell death caused by Bak overexpression. As shown in Fig. 2C, VK2 treatment resulted in the appearance of a slower-migrating band of FLAG-Bak, suggesting that the upper band represents a posttranslationally modified isoform of Bak. Although Bak is known to be phosphorylated in undamaged cells, such a significant mobility change has not been observed (Fox et al., 2010). Moreover, CHX did not change its mobility (Fig. 2, B and C), suggesting that this modification is specific to VK2-induced apoptosis.

Next, we investigated the possibility that VK2 may directly interact with Bak, because such an interaction would facilitate posttranslational modification, possibly by an allosteric mechanism. HL60 cell cytoplasmic extracts were subjected to affinity purification using ferrite-glycidyl methacrylate beads (Sakamoto et al., 2009) with or without immobilized VK2 (Supplementary Fig. 1). Bound proteins were eluted from the beads with SDS and subjected to immunoblot analysis. The results showed that Bak specifically bound to the VK2-beads,

while Bax did not (Fig. 2D). To confirm that this interaction is direct, we performed affinity purification using purified recombinant proteins synthesized *in vitro*. As shown in Fig. 2E, HA-FLAG-tagged Bak, but not HA-FLAG-tagged Bax, bound to the VK2-beads, confirming that VK2 selectively binds to Bak and induces its mobility shift.

These findings prompted us to investigate the role of Bak in VK2-induced apoptosis. HeLa cells were transfected with siRNA targeting Bak or Bax, and were then treated with VK2 for 48 h, and cell viability and cyt c release were examined. Depletion of Bak decreased VK2-induced growth inhibition, whereas depletion of Bax did not (Fig. 2F). In addition, VK2-induced cyt c release was suppressed by depletion of Bak, whereas it was enhanced by depletion of Bax (Fig. 2G). Therefore, although both Bak and Bax are activated by VK2 (Fig. 1E), activation of Bax alone does not result in apoptosis, and Bak is necessary and sufficient for VK2-induced apoptosis.

**VK2 2,3-Epoxy Is Coupled to Bak through Its Cysteine-166 Residue.** Given the importance of Bak in VK2-induced apoptosis, we used mass spectrometry (MS) to identify the specific VK2-induced posttranslational modification of Bak. HeLa cells transiently overexpressing FLAG-Bak were treated with VK2, and FLAG-Bak was immunoprecipitated with anti-FLAG antibody and subjected to liquid chromatography-electrospray ionization MS analysis. As shown in Fig. 3A, VK2 treatment caused a  $459.9 \pm 2.1$  Da shift in the molecular mass of FLAG-Bak (the error was estimated from experimental and theoretical values of the molecular mass of FLAG-Bak). Because the shift was close to the molecular weight of VK2 (444.7 Da), we focused on its metabolites. As shown in Fig. 3B, of the two main VK2 metabolites, VK2 hydroquinone (VK2-H2, 446.7 Da) and VK2 2,3-epoxide (VK2-O, 460.7 Da) (Oldenburg et al., 2008), the latter is particularly close to the molecular weight shift observed ( $459.9 \pm 2.1$  Da). Coincidentally, a number of reports have demonstrated that the epoxy ring of small molecules is opened to form a covalent bond with a cysteine residue of its target proteins under physiological conditions (Watanabe et al., 2006), suggesting that VK2-O, but not VK2 or VK2-H2, may form a covalent bond with Bak. Therefore, we

considered the possibility that VK2-O reacts with a cysteine residue of Bak.

Because Bak has only two cysteine residues, i.e., Cys-14 and Cys-166, we constructed Bak mutants in which one or both of the cysteine residues were mutated to alanine. These mutations did not appreciably affect the affinity between VK2 and Bak (Supplementary Fig. 2). HeLa cells transfected with FLAG-Bak wild type (WT) or one of its mutants were treated with VK2 and the pan-caspase inhibitor zVAD-fmk. As shown in Fig. 3C, C166A and C14A/C166A did not show a VK2-induced mobility shift, indicating that Cys-166 is necessary for VK2-induced modification. Next, we examined the redox status of cysteine residues using a FD5M labeling assay. After VK2 treatment, FLAG-Bak was immunoprecipitated and labeled with FD5M, which specifically reacts with free thiol groups. WT, but not C14A/C166A, was labeled by FD5M (Fig. 3C). Remarkably, the faster-migrating form of C14A was labeled by FD5M, but the slower-migrating form of C14A was not, indicating that FD5M labeling of Cys-166 was prevented by its reaction with VK2. These data suggested that VK2 specifically reacts with the thiol group of Bak Cys-166.

As Bak also interacts with VK2 noncovalently (Fig. 2E), we then mapped the noncovalent binding region of Bak using a series of point mutants, and found that the R169A and W170A mutations of Bak weakened its interaction with VK2 (Fig. 3D). Moreover, the double point mutant R169A/W170A had extremely low VK2-binding activity. These results suggest that VK2 and VK2-O interact with Bak through its Arg-169 and Trp-170 residues noncovalently, and that VK2-O further forms a covalent bond with the adjacent Cys-166 residue.

**Covalent Attachment of VK2 Is Correlated with Apoptosis Induction.** As shown in Fig. 2B, the appearance of the VK2-modified form of Bak seems to coincide with VK2-induced apoptosis. To study this point further, we used the free radical scavenger alpha-tocopherol, based on a previous study showing that VK2 induces the production of reactive oxygen species (ROS) and that VK2-induced apoptosis is inhibited by alpha-tocopherol (Shibayama-Imazu et al., 2006). We first examined ROS generation in

HL60 cells using flow cytometry and APF. APF is a ROS-sensitive probe that is oxidized by intracellular ROS into a highly fluorescent compound and has been used to detect the generation of ROS during apoptosis (Nakazato et al., 2007; Inoue et al., 2009). As shown in Fig. 4A, ROS production was induced in HL60 cells after 24 h incubation with VK2 and was partially suppressed by the presence of alpha-tocopherol.

Next, we investigated the effect of alpha-tocopherol on the generation of the VK2-modified form of Bak. Alpha-tocopherol inhibited VK2-induced cleavage of PARP and covalent attachment of VK2 to Bak in a similar concentration-dependent manner (Fig. 4B). Taken together, these results indicate a strong correlation between covalent attachment of VK2 to Bak and VK2-induced apoptosis.



## DISCUSSION

In this study, we investigated the molecular mechanism of VK2-induced apoptosis. The results obtained using HL60 cells and isolated mitochondria indicate that VK2 induces mitochondrial-mediated apoptosis and activates caspase-3 (Fig. 1B-D). Consistent with these results, previous studies show that VK2 induces apoptosis through the mitochondrial-mediated pathway and the activation of caspase-3 in human myeloma cells and HL60 cells (Tsujioka et al., 2006; Yokoyama et al., 2008). Moreover, although VK2 activated both Bak and Bax (Fig. 1E), VK2-induced apoptosis was abrogated by the knockdown of Bak, but not of Bax (Fig. 2, F and G). Furthermore, VK2 was shown to directly bind to Bak, but not to Bax (Fig. 2E). Bak is known to be suppressed by antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL. Concordantly, the overexpression of Bcl-2 or Bcl-xL in HL60 or HeLa cells abrogated VK2-induced apoptosis (Yokoyama et al., 2008 and our unpublished data). Thus, only Bak is necessary and sufficient for VK2-induced apoptosis and is a molecular target in VK2-induced apoptosis.

The proapoptotic functions of Bak and Bax are generally considered to be highly redundant. Whereas *Bak*<sup>-/-</sup> mice are fertile and do not have any gross morphological defects, *Bax*<sup>-/-</sup> mice have minor phenotypic abnormalities that are characteristic of defects in apoptosis, and *Bak*<sup>-/-</sup>*Bax*<sup>-/-</sup> mice have multiple developmental defects and often die perinatally (Knudson et al., 1995; Lindsten et al., 2000). These studies indicate that Bak and Bax have overlapping roles in the regulation of apoptosis and that Bax, but not Bak, has non-redundant functions. Thus, the specific role of Bak presented in the current study is unprecedented, to our knowledge. Although it is still unclear why VK2-induced apoptosis is specifically mediated by Bak, it is tempting to speculate that the covalent attachment of Bak by VK2 is involved in this process. Because the potency of VK2 as an apoptosis-inducing agent is weak, unmodified Bax and/or Bak may be insufficient to induce apoptosis, and the modification of Bak may facilitate apoptosis.

In addition to its anticancer effects, VK2 functions in blood coagulation and bone homeostasis. We speculate that these effects are mediated by different molecular targets, as

discussed below. First, VK2 is an essential cofactor for GGCX which catalyzes the  $\gamma$ -carboxylation of its substrates. As a number of blood coagulation factors require  $\gamma$ -carboxylation for their function (Stafford, 2005), GGCX is clearly important for the blood coagulation function of VK2. Moreover, osteocalcin, which plays important roles in bone formation, is also activated by  $\gamma$ -carboxylation (Hauschka et al., 1975); therefore, GGCX is also involved in bone homeostasis. Second, VK2 binds to SXR and activates the transcription of its target genes related to collagen accumulation (Tabb et al., 2003; Ichikawa et al., 2006). Hence, VK2 likely contributes to bone homeostasis not only through GGCX but also through SXR. Third, HSD17B4, an enzyme converting E2 to E1, was also identified as a VK2-binding protein (Otsuka et al., 2005), although the role of HSD17B4, if any, in VK2 biology is not well understood. Fourth, this study identified Bak as a molecular target of VK2-induced apoptosis. To our knowledge, there are few reports demonstrating the relations among GGCX, SXR, and Bak, which suggests that these factors mediate VK2-induced effects independently. Nevertheless, the fact that VK2-O, converted from VK2 by the vitamin K cycle, covalently attaches to Bak during VK2-induced apoptosis indicates that the vitamin K cycle may be related to Bak-mediated apoptosis.

Here, we found that VK2-O, an intracellular metabolite of VK2, covalently binds to the Cys-166 residue of Bak (Fig. 3C). Several lines of evidence suggest that this modification contributes to VK2-induced apoptosis. First, the VK2-bound form of Bak accumulated with time, and apoptotic phenotypes were observed only after its accumulation (Fig. 1B and 2B), suggesting that the accumulation of modified Bak to a critical level is necessary for VK2-induced apoptosis. Second, in support of this view, the covalent attachment of VK2-O and apoptosis induction were suppressed by alpha-tocopherol in HL60 cells (Fig. 4B), suggesting that these processes are coordinately controlled by the intracellular redox status. Concordantly, a previous study demonstrated that VK2-induced apoptosis in TYK-nu cells was suppressed by anti-oxidants such as alpha-tocopherol, Trion, and N-acetyl-L-cysteine (Shibayama-Imazu et al., 2006). Third, although VK2 has been shown to induce the generation of ROS (Shibayama-Imazu et al., 2006 and Fig. 4A), ROS production was not

abrogated by the knockdown of Bak (unpublished data). It is therefore likely that VK2-induced ROS generation occurs upstream of Bak and induces apoptosis in a Bak-dependent manner, possibly by controlling its modification by VK2-O. Fourth, a recent study in human prostate cancer LNCaP cells demonstrated that VK2-induced anticancer effects are abrogated by GGCX knockdown (Akamatsu et al., 2012), which probably inhibits the generation of VK2-O and prevents the covalent modification of Bak. Thus, we established a strong correlation between the covalent attachment of VK2-O and apoptosis induction. However, our study did not provide direct evidence that the covalent modification of Bak is actually responsible for VK2-induced apoptosis. The establishment of a cell line in which endogenous Bak is substituted by the covalent binding-deficient mutant C166A and its comparison with the parental cell line may clarify this issue. Such rescue experiments have been attempted but not completed, because Bak overexpression causes substantial cell death, and tight control of exogenous Bak gene expression is required to establish the above-mentioned cell line.

We identified the covalent attachment of VK2-O to a cysteine residue (VK2ation) for the first time, although the reaction mechanism remains unclear. Based on synthetic chemistry, VK2ation may proceed without any auxiliary factor under physiological conditions. However, we have not successfully reconstituted the VK2ation of Bak using purified Bak and either VK2 or VK2-O *in vitro* (unpublished data), suggesting that other factors may be required for the VK2ation process. In this respect, alpha-tocopherol treatment abrogated the VK2ation of Bak (Fig. 4B), supporting the notion that factors other than Bak may be required for its VK2ation in the cell. ROS may control VK2ation directly or indirectly through other molecules. Indirect regulation is supported by results showing that H<sub>2</sub>O<sub>2</sub>-induced oxidative stress upregulated the expression of the vitamin K epoxide reductase complex, subunit 1-like 1 (VKORC1L1) gene, a component of the vitamin K cycle, and increased its activity (Westhofen et al., 2011). Hence, alpha-tocopherol treatment may conversely slow down the vitamin K cycle, inhibit VK2-O synthesis, and thereby abrogate the VK2ation of Bak indirectly. Regardless of the mechanism involved, this study revealed a

new posttranslational modification, VK2ation, which could potentially occur in other VK2 target molecules other than Bak.

In the present study, we identified Bak as a molecular target of VK2-induced apoptosis. Moreover, this study suggested the possibility that Bak may serve as a molecular target of anticancer drugs. Proapoptotic Bcl-2 family proteins, such as Bak and Bax, are known to be suppressed by antiapoptotic proteins of the same family, such as Bcl-2 and Bcl-xL. In fact, pan-antiapoptotic Bcl-2 family inhibitors such as obatoclax and navitoclax are being tested in clinical trials (O'Brien et al., 2009; Gandhi et al., 2011). Moreover, small molecules that directly bind to and activate Bax *in vitro* have been developed and are expected to be used for cancer treatment (Gavathiotis et al., 2012). Thus, members of the Bcl-2 family are promising targets for cancer therapy. Our findings are in line with this notion and may contribute to the development or improvement of anticancer drugs targeting Bcl-2 family proteins.

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## **AUTHORSHIP CONTRIBUTIONS**

Participated in research design: Karasawa, Azuma, Imai, Miyazawa, and Handa.

Conducted experiments: Karasawa, and Azuma.

Contributed new reagents or analytic tools: Kasama, Sakamoto, and Kabe.

Performed data analysis: Karasawa, Azuma, and Kasama.

Wrote or contributed to the writing of the manuscript: Karasawa, and Yamaguchi.

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## FOOTNOTES

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## FIGURE LEGENDS

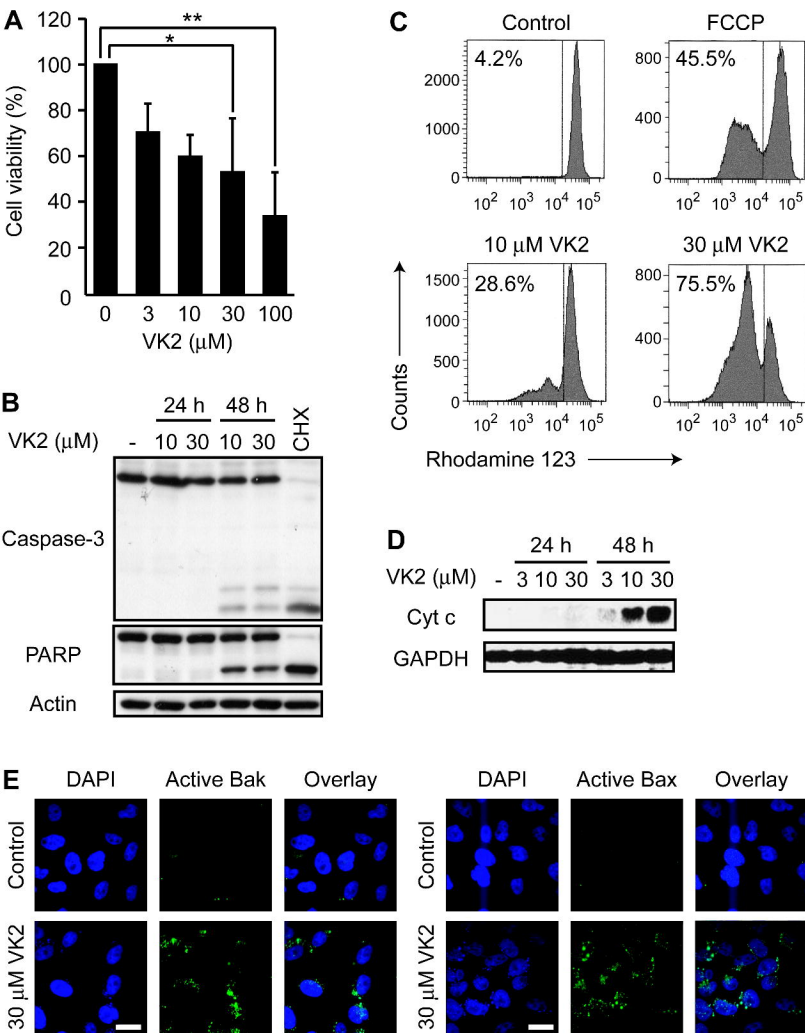
**Fig. 1.** VK2 induces mitochondrial-mediated apoptosis. A, HL60 cells were treated with the indicated concentrations of VK2 for 48 h. After treatment, cell viability was determined using Cell Count Reagent SF. Each error bar represents the mean  $\pm$  standard deviation (SD) of three independent experiments. \*\*,  $p < 0.01$  and \*,  $p < 0.05$  (Student's t test). B and D, HL60 cells were treated with VK2 or CHX and harvested for immunoblotting. C, HL60 cells were treated with VK2 for 48 h or FCCP for 4 h, and mitochondrial membrane potential was measured by flow cytometry using Rhodamine 123. E, HeLa cells were treated with VK2 for 48 h, and the active forms of Bak and Bax were visualized using isoform-specific antibodies. Scale bar represents 10  $\mu$ m.

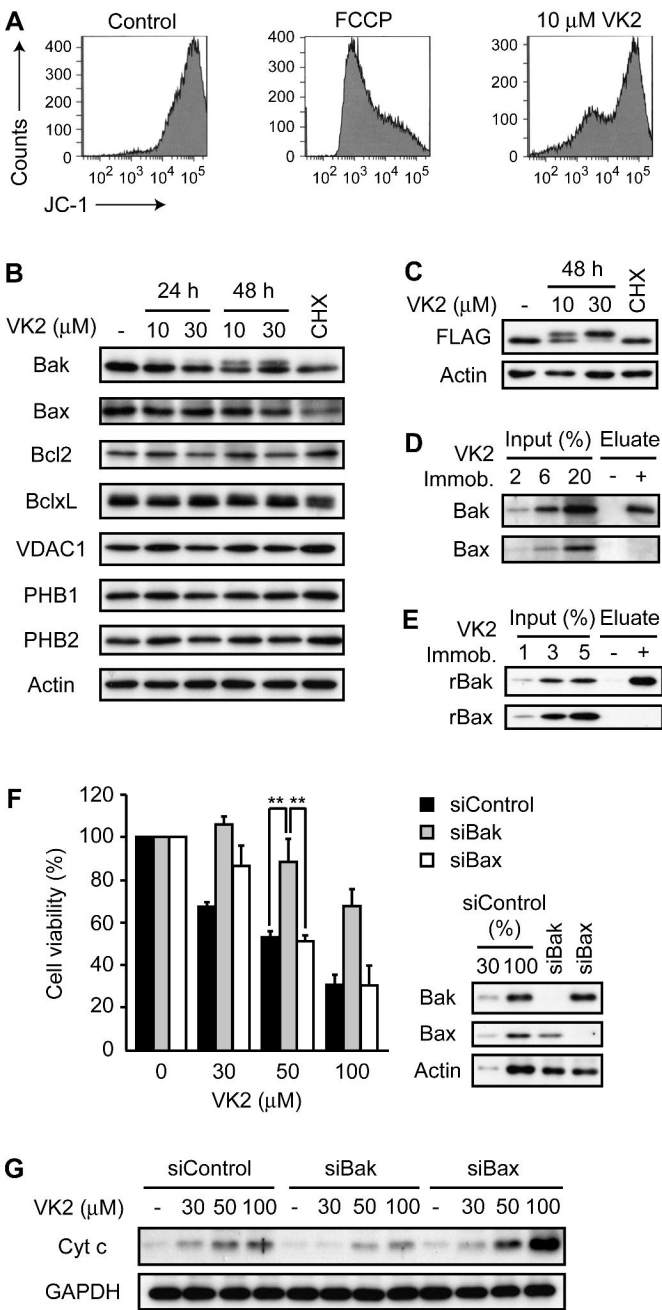
**Fig. 2.** Bak interacts with VK2 and mediates VK2-induced apoptosis. A, Isolated mitochondria were incubated with 10  $\mu$ M VK2 or 0.1 mg/mL FCCP for 3 h. Mitochondrial membrane potential was measured by flow cytometry using JC-1. B, HL60 cells were treated with VK2 or CHX and harvested for immunoblotting. C, HeLa cells expressing FLAG-Bak were treated with the indicated concentrations of VK2 for 48 h or with CHX for 24 h and harvested for immunoblotting. D, VK2-immobilized beads (+) or control beads (-) were incubated with HL60 cytoplasmic extracts. Bound proteins were subjected to immunoblotting using the indicated antibodies. E, Purified recombinant Bak and Bax (rBak and rBax) were individually incubated with VK2-immobilized beads (+) or control beads (-). Input and eluate materials were analyzed by immunoblotting against anti-FLAG antibody. F and G, HeLa cells were transfected with siRNA targeting Bak or Bax or control siRNA. Cells were treated for 48 h with VK2 before being harvested for cell proliferation assays or immunoblotting. Each error bar represents the mean  $\pm$  SD of three independent experiments. \*\*,  $p < 0.01$  (Student's t test).

**Fig. 3.** VK2 2,3-epoxide is coupled to Bak through its Cys-166 residue. A, HeLa cells expressing FLAG-Bak were treated with 30  $\mu$ M VK2 for 48 h or left untreated, and

FLAG-Bak was immunoprecipitated for LC-MS analysis. Parent peaks of unmodified and modified Bak are shown. B, Schematic representation of VK2 metabolism in mammals. VKORC1, vitamin K epoxide reductase complex, subunit 1; VKORC1L1, VKORC1-like 1; GGCX,  $\gamma$ -glutamyl carboxylase; VK2-H2, vitamin K2 hydroquinone. C, HeLa cells expressing FLAG-Bak wild type (WT) or one of its mutants were incubated with or without 30  $\mu$ M VK2 for 48 h. Cell lysates were immunoblotted with the indicated antibodies (input). Alternatively, FLAG-Bak proteins were immunoprecipitated, and free cysteine residues were labeled with FD5M. The reaction mixtures were subjected to SDS-PAGE followed by immunoblotting or fluorescence assay (FD5M-labeled). D, Purified recombinant FLAG-Bak wild type or one of its mutants were incubated with VK2-immobilized beads (+) or control beads (-). Input and eluate materials were analyzed by immunoblotting.

**Fig. 4.** Covalent attachment of VK2 to Bak is correlated with apoptosis induction. A, HL60 cells were treated with VK2, alpha-tocopherol ( $\alpha$ -toco), or both for 24 h. Then, ROS production was measured by flow cytometry using APF. The values represent the populations of ROS-high (P1-gated) cells. Gray lines indicate the control cells. B, HL60 cells were incubated with or without VK2 and alpha-tocopherol for 48 h and were harvested for immunoblotting.

**Figure 1**

**Figure 2**



**Figure 3**