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The role of PKC isoforms in the inhibition of NF-κB activation by vitamin K2 in human hepatocellular carcinoma cells☆

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Abstract

Vitamin K (VK) has diverse protective effects against osteoporosis, atherosclerosis and carcinogenesis. We recently reported that menatetrenone, a VK2 analogue, suppressed nuclear factor (NF)-κB activation in human hepatoma cells. Although NF-κB is regulated by isoforms of protein kinase C (PKC), the involvement of PKCs in VK2-mediated NF-κB inhibition remains unknown. Therefore, the effects of VK2 on the activation and the kinase activity of each PKC isoform were investigated. The human hepatoma Huh7 cells were treated with PKC isoform-specific inhibitors and/or siRNAs against each PKC isoform with or without 12-*O*-tetradecanoylphorbol-13-acetate (TPA). VK2 inhibited the TPA-induced NF-κB activation in Huh7 cells. NF-κB activity was inhibited by the pan-PKC inhibitor Ro-31-8425, but not by the PKCα-specific inhibitor Gö6976. The knockdown of individual PKC isoforms including PKCα, δ and ε showed only marginal effects on the NF-κB activity. However, the knockdown of both PKCδ and PKCε, together with treatment with a PKCα-specific inhibitor, depressed the NF-κB activity. VK2 suppressed the PKCα kinase activity and the phosphorylation of PKCε after TPA treatment, but neither the activation nor the enzyme activity of PKCδ was affected. The knockdown of PKCε abolished the TPA-induced phosphorylation of PKD1, and the effects of PKD1 knockdown on NF-κB activation were similar to those of PKCε knockdown. Collectively, all of the PKCs, including α, δ and ε, and PKD1 are involved in the TPA-mediated activation of NF-κB. VK2 inhibited the NF-κB activation through the inhibition of PKCα and ε kinase activities, as well as subsequent inhibition of PKD1 activation.

Keywords: Vitamin K2; NF-κB; PKC; PKD1; Liver cancer

1. Introduction

Vitamin K (VK), an essential nutrient for the production of functional blood coagulation factors, has emerged as an important factor protecting against diverse diseases such as osteoporosis, atherosclerosis and several types of neoplasms, including hepatocellular carcinoma (HCC) [1–4]. The K vitamins can be divided into two groups: naturally produced VK1 (phylloquinone) and VK2 (menaquinone), and chemically synthesized VK3 (menadione). VK1 is contained in a wide range of plants; VK2 is of microbial origin and is widely distributed in leafy vegetables, eggs, cheese, meats and fermented soybeans.

Previously, several VK analogues have been reported to possess antitumor activity against hematological malignancies and solid tumors, such as HCC [3–7]. We and others have demonstrated that the administration of menatetrenone, a VK2 analogue, suppresses the recurrence of HCC after curative ablation therapy [8] or the development of HCC from cirrhotic livers [9] in the clinical setting. VK2 also suppresses the development and proliferation of HCC in *in vivo* animal models [10–12]. Recently, we have revealed that VK2 inhibits the growth of HCC cells by suppressing cyclin D1 expression through the inhibition of nuclear factor (NF)-κB activation [13], as well as via inhibition of the expression of matrix metalloproteinases (MMPs) that contain an NF-κB binding motif in their promoter region and are considered to be involved in both the invasion and metastasis of carcinoma cells [14,15].

Protein kinase C (PKC), one of the phospholipid-dependent serine/threonine kinase families, is subdivided into conventional PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKCs [16,17]. When cells are treated with activators, including tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cPKCs and nPKCs are activated and play pivotal and key regulatory roles in a number of cellular functions, such as cellular growth and migration, accompanied by changes in gene expression [16,17]. PKCs have been reported to participate in the activation of AP-1 and NF-κB and increase their subsequent gene expression [18–20]. We have shown that VK2 suppressed the expression of MMPs, along with the inhibition of TPA-

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induced activation of NF-κB [13,14], suggesting that VK2 might act as a PKC inhibitor. These results prompted us to clarify the role of PKCs in VK2-mediated inhibition of NF-κB activation. In this report, we have examined the role of each PKC isoform in the activation of NF-κB and have also evaluated the effects of VK2 on the activation of individual PKC isoforms and their kinase activities.

2. Materials and methods

2.1. Cell lines and reagents

Huh7 cells were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. Menatetrenone, a VK2 analogue, was provided by Eisai Co. (Tokyo, Japan) and dissolved in ethanol. The PKC inhibitors Ro-31-8425 and Gö6976 were from Calbiochem (San Diego, CA, USA), and TPA was from Sigma-Aldrich. SiRNAs against the PKCα, δ and ε isoforms (HP validated, S100301308, S102660539, S100287784, respectively), PKD1 (S100301350) and Allstar negative control siRNA (S1027281) were from Qiagen (Heiden, Germany). Human PKCα plasmids, pHACE-PKCα-WT (wild type), -DN (dominant negative), -δNPS (constitutive active) and -CAT (constitutive active) were kindly denoted by Dr. Joe-Won Soh, Laboratory of Signal Transduction, Department of Chemistry, Inha University, Korea [21]. Anti-PKCα, δ, ε antibodies were obtained from Santa Cruz, CA, USA. Anti-phospho-PKCα (Thr497, Thr638), anti-phospho-PKCδ (Thr505, Ser643), anti-phospho-PKCε (Ser729), anti-phospho-PKD1 (Ser744), anti-PKD1 and anti-phospho-IκBα (Ser32/36) antibodies were the products of Cell Signaling Technology (Beverly, MA, USA). Anti-human β-actin antibody was purchased from Biomedical Technologies (Stoughton, MA).

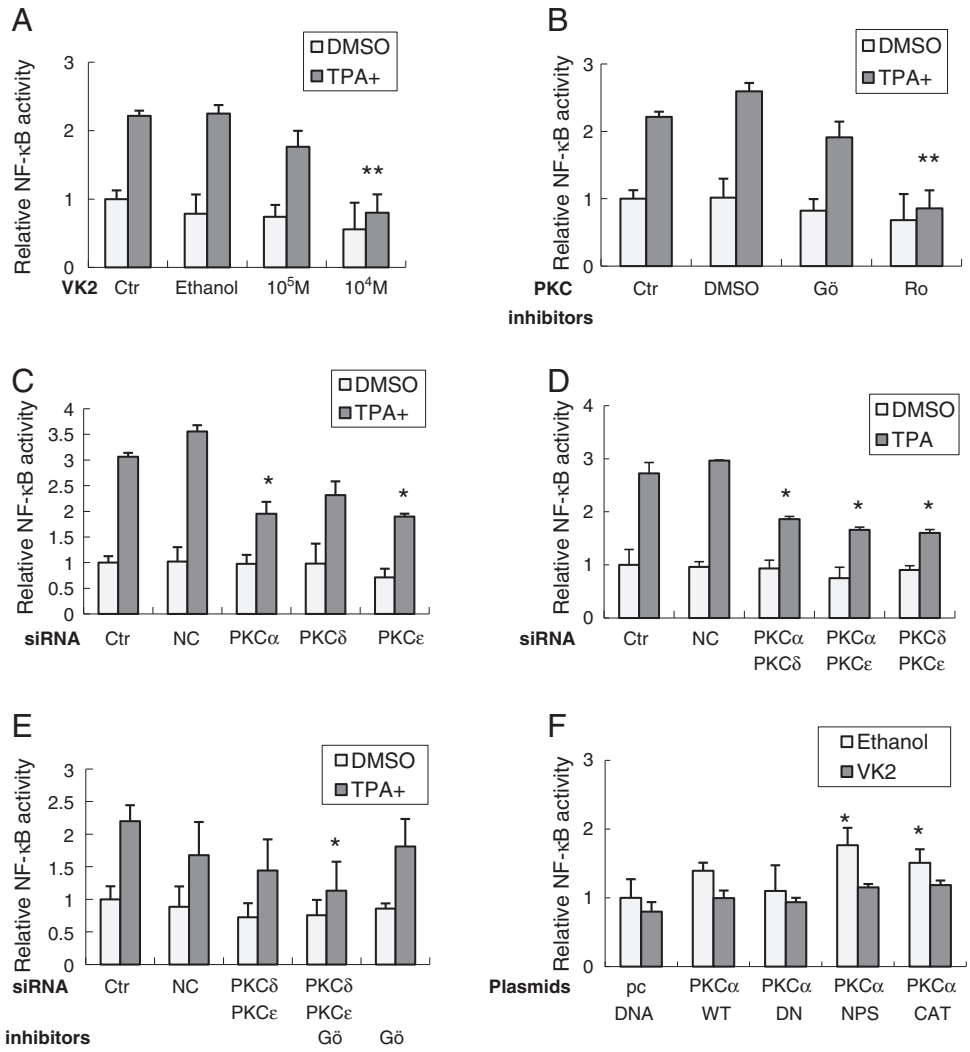

Fig. 1. Effect of VK2 and PKCs on the NF-κB activity in the NF-κB reporter gene transfectants. (A) VK2 suppressed the basal and TPA-induced NF-κB transcriptional activity in a dose-dependent manner in Huh7 cells. Huh7 NF-κB reporter gene transfectants in 48-well plates were cultured with or without the indicated concentrations of VK2 for 24 h, then treated with or without TPA(50 nM) for 3 h and subjected to luciferase assay as described in Materials and Methods. (B) PKC inhibitors showed inhibitory effect on NF-κB transcriptional activity in Huh7 cells. Huh7 transfectants were cultured in the presence or absence of PKC inhibitors as indicated in the figure for 24 h and subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (C) Individual siRNA-mediated knockdown of PKCs inhibited NF-κB transcriptional activity. Huh7 transfectants were transfected as described in Materials and Methods with siRNA of PKC isoforms as indicated in the figure, cultured for 24 h, and subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (D) Co-siRNA-mediated knockdown of PKCs significantly inhibited NF-κB transcriptional activity. Huh7 transfectants in 48-well plates were co-transfected with two siRNAs (10 nM for each) against two different PKCs or with negative control siRNA (20 nM), cultured for 24 h and subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (E) Combination of knockdown of PKCδ and ε and treatment with PKCα inhibitor Gö6976 suppressed the NF-κB activity to a similar level as the pan-PKC inhibitor did. Huh7 transfectants in 48-well plates were co-transfected with PKCδ and ε siRNA as described in panel (D). After culturing for 24 h, cells were treated with Gö for 24 h and then subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (F) Effect of various PKCα mutants on the NF-κB activity. Huh7 transfectants cultured in 48-well plates were transfected with PKCα mutant plasmids as described in Materials and Methods. At 24 h after transfection, 10^{-4} M of VK2 was added to the wells and cultured further for 24 h. Then, the cells were subjected to luciferase assay immediately. Columns, mean obtained from at least three independent experiments; bars, S.D.; *P<.05; **P<.01 (Student's t test); Ctr, no treatments; NC, negative siRNA; Gö, PKCα inhibitor Gö6976; Ro, pan-PKC inhibitor Ro-31-8425. PKCα WT, pHACE-PKCα wild type; PKCα DN, pHACE-PKCα dominant negative; PKCα NPS and PKCα CAT, pHACE-PKCα constitutive active form pcDNA, pcDNA3.1 vector as control.

2.2. Stable transfectants of the NF-κB reporter gene and the luciferase reporter gene assay

To obtain stable NF-κB reporter gene transfectants, pGL4.17NF-κB, a NF-κB reporter plasmid which was produced by conjugating the NF-κB promoter sequence of pNF-κB Luc (Clotech, CA, USA) with luceferase gene in pGL4.17[Luc2/Neo] vector (Promega, Madison, WI) or the vector alone (as a control) was introduced into Huh7 cells using Lipofectamine 2000 (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The transfected cells were treated with 500 ng/ml G418 for 2 weeks before selection. Clones expressing the NF-κB reporter gene were selected and subjected to further analysis. The NF-κB transcriptional activity was detected by a luciferase assay using the Single-Luciferase Reporter Assay System according to the method described by the supplier (Promega, Madison, WI, USA). Transfectant cells were seeded onto 48-well plates at 2×10^3 per well in DMEM with 10% FBS and incubated until 80% confluent at 37°C before use. After incubation with different concentrations of VK2 or PKC isoform-specific inhibitors for 24 h, 50 nM TPA/dimethyl sulfoxide (DMSO) in serum-free medium was added to the wells for 3h of further culture. Then, the cells were washed twice with phosphate-buffered saline (PBS) and carefully lysed in 1× passive lysis buffer (Promega, Madison, WI, USA). The cell extracts were immediately assayed for single luciferase activity using a Berthold Luminometer (MLR-100 Micro Lumino Reader, Corona Electric, Ibaragi, Japan). The amount of protein in cell extracts was determined using a Pierce660nm protein assay system (Thermo Scientific, USA). The luciferase activity was normalized by the protein amount.

2.3. SiRNA-mediated knockdown of PKCs and PKD1, and transfection of PKCα plasmids

The cells that were stably transfected with the NF-κB reporter gene were seeded onto 48-well plates at 2×10^3 cells per well in DMEM with 10% FBS and incubated until 80% confluent at 37°C. Next, the cells were replaced with new DMEM containing 10% FBS without antibiotics after being washed twice with DMEM, followed by the addition of 0.2 ml of OPTI-MEM Reduced Medium (Life Technologies, Rockville, MD, USA) containing the specific siRNA and Lipofectamine RNAiMAX (Life Technologies, Rockville, MD, USA) complex prepared according to the manufacturer's protocol. Lipofectamine 2000 (Life Technologies) was used for the transfection of PKCα mutant plasmids. After 24 or 48 h of incubation, the transfected cells were treated with TPA in DMEM without FBS for 3 h and subjected immediately to a single luciferase assay.

2.4. Western blotting

The protein expression of PKC isoforms was investigated by a Western blotting analysis. The cells that were cultured under various conditions were collected and lysed with sodium dodecyl sulfate (SDS) buffer [50 mM Tris (pH 6.8), 2.3% SDS and 1 mM phenylmethanesulfonyl fluoride]. The cell debris was eliminated by centrifugation at 12,000*g* for 10 min, and the supernatant was collected. After measuring the protein concentration with a protein assay kit (Bio-Rad, Hercules, CA, USA), a proper amount of protein was mixed with SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked overnight at 4°C with 0.1% Tween 20 and 5% skim milk in PBS. The membranes were then incubated with the primary antibody for 1 h at room temperature or overnight at 4°C. The membranes were washed three times with 0.1% Tween 20 in PBS and stained with horseradish-peroxidase-conjugated secondary antibodies. All immunoblots were detected using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. For the detection of phospho-PKCs, Tris-buffered saline was used instead of PBS.

2.5. Kinase assay

The PKC kinase assay was performed using the Promega Kit according to the manufacture's official protocol with minor modifications. Huh7 cells were plated at a

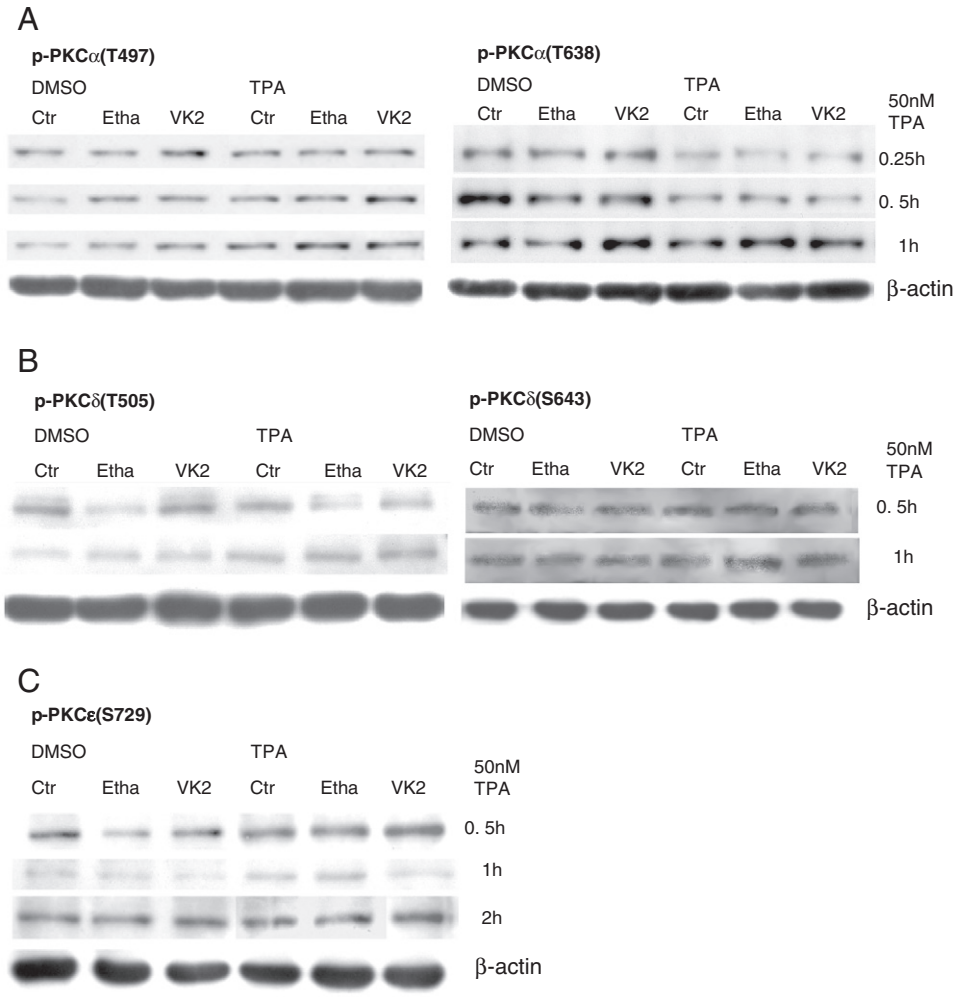

Fig. 2. VK2 effects on PKC activation in Huh7 cells. Huh7 cells were cultured in 3.5-cm dishes. After 24 h treatment with or without 10^{-4} M concentrations of VK2 or the VK2 solvent ethanol as a control, the cells were treated with 50 nM TPA or DMSO for the indicated time depending on the target protein. Cell extracts were analyzed by Western blotting using PKCs specific antibodies. Ctr, no treatments; Etha, ethanol.

density of 2×10^5 per 6-cm dish and were incubated at 37°C. After 72 h in culture, rat HA-tagged-PKC isoform plasmids [22] were introduced into the cells with Lipofectamine 2000 according to the manufacture's protocol. After 10^{-4} M VK2 was added, the cells were further cultured for 24 h, followed by 50 nM TPA treatment for 15 min in the case of PKCa and 1 h in the case of PKCd and PKCe. After cells were harvested with ice-cold PBS, they were sonicated in 1× cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin] in an ice-cold bath for 10 s. The lysates were centrifuged for 10 min at 14,000*g* at 4°C, and the supernatant was transferred to a new tube. The samples were then precipitated with 10–20 μl HA-tag agarose (MBL, Nagoya, Japan) with gentle rotating for 1 h at 4°C. Pellets were collected by centrifugation for 10 s at 2500*g* at 4°C and were washed three times with 500 μl of 1× washing cell lysis buffer, which was the same as the lysis buffer except that it contained 0.1% Triton X-100. The pellets were divided into five equal parts and subjected to the kinase assay according to the manufacture's protocol. One tube was subjected to a Western blotting analysis for the determination of the amount of PKC protein by determining the density of the PKC bands with the NIH ImageJ 1.41 software program (Bethesda, MD, USA).

2.6. Statistical analysis

Differences were analyzed using Student's *t* test, and $P<.05$ was considered to be significant. All experiments were done at least three times. The data are shown as the means±S.D.

3. Results

3.1. Effects of VK2 and PKCs on the NF-κB activity as determined by the NF-κB reporter gene transfectants

To investigate the mechanism responsible for the effects of VK2 on the inhibition of NF-κB transcriptional activity in Huh7 cells, a number of Huh 7 clones that were stably transfected with a NF-κB reporter gene were picked up successfully. Four of the clones, #4, #5, #8 and #14, were investigated, and similar results were observed for all four clones. As shown in Fig. 1A, TPA significantly stimulated NF-κB transcriptional activity in Huh7 cells, and VK2 abrogated the TPA-induced NF-κB transcriptional activity in a dose-dependent manner, in addition to weakly inhibiting the basal NF-κB transcriptional activity. The solvent used for VK2, ethanol, did not show any significant suppression, confirming that the TPA-induced activation of PKCs was involved in the NF-κB activation and that VK2 might inhibit NF-κB activation through the suppression of PKCs in Huh7 cells.

Various PKC isoforms, including PKCα, δ, ε, ι and ζ, are expressed in Hep3B hepatoma cells [23]. We confirmed by reverse transcriptase polymerase chain reaction that Huh7 cells also express these isoforms (data not shown). Of these PKCs, α (cPKC), δ and ε (nPKC) are activated by TPA. To determine which PKC isoform(s) was(were) involved in activating the NF-κB transcriptional activities, we first utilized specific inhibitors of PKCs, as shown in Fig. 1B. A pan-PKC inhibitor, Ro-31-8425 (100 nM), significantly inhibited TPA-induced NF-κB transcriptional activity. However, a PKCα inhibitor, Gö6976 (10 nM), only slightly suppressed the TPA-induced NF-κB activity. Next, we performed a siRNA-mediated PKC knockdown in the Huh7 cells. As shown in Fig. 1C and D, we observed that knockdown of individual PKC isoforms PKCα and ε significantly deceased the TPA-induced NF-κB luciferase activity, but knockdown of PKCδ only slightly decreased the activity (Fig. 1C). Furthermore, the simultaneous knockdown of two of these three PKC isoforms in the cells was still not sufficient to suppress the activity to the same level as that induced by the pan-PKC inhibitor or 10^{-4} M VK2 (Fig. 1D). These results suggested that all three of these PKC-isoforms, PKCα, δ and ε, may participate in the activation of NF-κB transcriptional activities.

To confirm this, we performed additional experiments combining siRNA-mediated knockdown of PKCs and treatment with a PKC inhibitor. At 24 h after co-transfection of cells with siRNAs against PKCδ and ε, 10 nM of the PKCα inhibitor (Gö6976) was added. After a further 24 h in culture, the cells were treated with DMSO/TPA for 3 h. When we used a combination of the knockdown of PKCδ and ε and Gö6976, we observed a similar level of suppression of the NF-κB activity as was observed for the pan-PKC inhibitor.

To further classify the PKCs involved, we employed various constitutively active forms of PKC enzymes. As shown in Fig. 1F, the constitutively active forms of PKCα, NPS and CAT, stimulated the activation of NF-κB in the absence of TPA more than the wild-type form (WT) did. In contrast, the dominant negative form (DN) showed no effect on the activation of NF-κB. Although constitutively active forms of PKCs δ and ε also were investigated, no significant changes were observed (data not shown). The results indicated that all of the TPA-induced PKC isoforms, α, δ and ε, are involved in the activation of NF-κB in Huh7 cells.

3.2. VK2 only partially change the phosphorylation levels of the PKC isoforms

To assess which PKC isoform(s) was (were) involved and whether the VK2 treatment affected its (their) activation or the activity itself, we performed a Western blotting analysis using various phosphorylation site-specific PKC antibodies. The phosphorylations of the activation loop (A-loop), turn motif (TM) and hydrophobic motif (HM) sites, which are thought to be involved in the activation of PKCs,

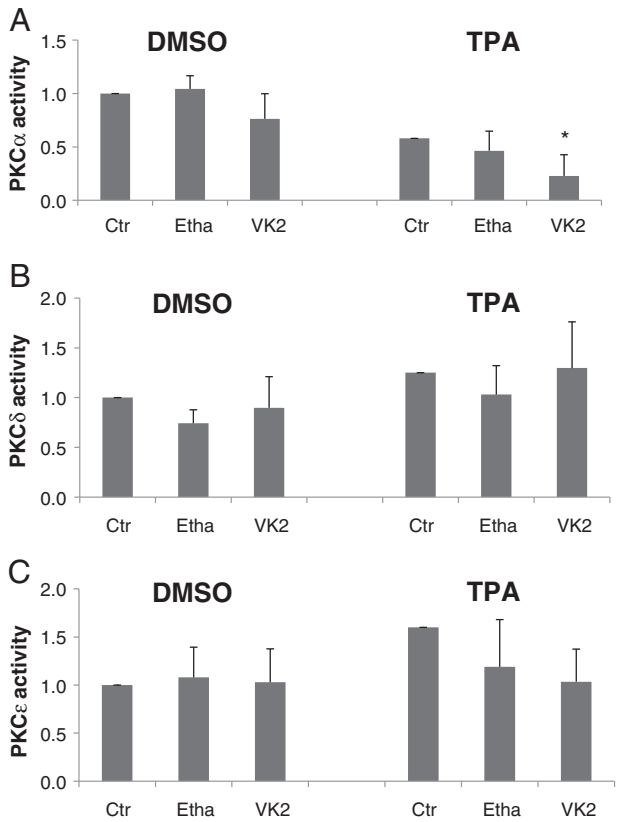

Fig. 3. Effects of VK2 on the enzyme activity of PKCs in Huh7 cells. PKC samples for enzyme assay were prepared as described in Materials and Methods, and PKC kinase activity of each sample was assayed at two different times of incubation when the activity linearly increased (usually at 15 and 30 min). (A) VK2 slightly suppressed PKCa activity at basal level (DMSO), while significantly at TPA-mediated level (TPA) in Huh7 cells. (B) VK2 inhibited PKCd activity at neither basal nor TPA-induced level. (C) VK2 did not suppress PKCe activity at both basal and TPA-induced levels. Columns are shown as relative enzyme activities when the activity obtained from the cells without any treatment is set at 1. The activity of each sample is a mean of data obtained from four independent cultures for (A), three independent cultures for (B), and four independent cultures for (C). Bars, S.D. * $P<.05$, compared with the activity of the enzyme obtained from the cells untreated with VK2 (Student *t* test). Ctr, control without treatment; Etha, treatment with the VK2 solvent ethanol; VK2, treatment with 10^{-4} M VK2.

were assayed. As shown in Fig. 2A, from 15 min to 1 h after treatment with TPA, after culturing with or without VK2, there were no clear effects on the phosphorylation of the Thr497 (A-loop) site. However, on the Thr638 (TM) phosphorylation site, after 15-min or 30-min treatments with 50 nM of TPA, PKCα showed significantly decreased phosphorylation in Huh7 cells, and these effects disappeared after 1 h of treatment. VK2 showed a limited effect on the Thr638 (TM) phosphorylation both at the basal level and in the TPA-treated state. As shown in Fig. 2B, TPA stimulated the phosphorylation of PKCδ at Thr505 (A-loop), but had a minimal effect on Ser643 (TM) after 1 h treatment. However, VK2 did not show any effect on either of the phosphorylation sites of PKCδ. TPA stimulated the phosphorylation of PKCε at Ser729 (HM) after 0.5 h and 1 h of treatments. At 1 h after treatment with TPA, this stimulation could be suppressed by VK2 (Fig. 2C). The expression levels of all PKCs were unchanged by the treatment (data not shown).

3.3. Effect of VK2 on PKC enzyme activity

Perhaps because of the limited phosphorylation site-specific antibodies for PKC that we used, we could not observe the inhibition of phosphorylation in any PKC induced by VK2 treatments. We next examined if the PKC enzyme activities were affected by VK2 treatment. HA-tagged PKC-plasmids were transiently introduced into Huh7 cells, then the HA-tagged PKCs were immunoprecipitated with an anti-HA antibody after VK2 and/or TPA treatments, and the phosphatidylserine- and diacylglycerol-stimulated PKC enzyme activity was determined *in vitro* by a kinase assay. TPA decreased the PKCα activity after 15 min of (Fig. 3A). The data may reflect the results of Western blot that the phosphorylation of the Thr638 was decreased with TPA treatment for 15 or 30 min (Fig. 2A). VK2 suppressed PKCα activity slightly at the basal level and significantly after TPA stimulation in Huh7 cells (Fig. 3A). VK2 suppression of PKCα activity was repeatedly observed in independent cultures, though the suppression levels were variable in the cultures. TPA stimulated the activities of PKCδ and ε after 1 h with 50 nM of TPA stimulation (Fig. 3B and C, respectively). Western blot analyses showed that the phosphorylation of PKCδ (Thr505) and PKCε (Ser729) was increased after 1-h treatment with TPA (Fig. 2B and C, respectively). VK2 did not show any inhibitory effects on the PKCδ activity at either the basal or TPA-induced level, as Fig. 3B showed. In PKCε, VK2 slightly suppressed the activity at the TPA-induced level, although it was not significant statistically (Fig. 3C). These results indicate that VK2 suppresses NF-κB activation through the inhibition of PKCα activity and also likely the activation of PKCε.

3.4. PKD1 is involved in the VK2-mediated inhibition of NF-κB activation through PKCs

Since PKCδ and ε have been shown to be the upstream factors of PKD1 (formerly called PKCμ) [24–26], PKD1 might be involved in the regulation of VK2-mediated NF-κB inhibition. As shown in Fig 4A, TPA stimulated the phosphorylation of PKD1 at Ser744 in A-loop, and VK2 inhibited the TPA-induced phosphorylation. The phosphorylation of IκBα at Ser 32/36 that is critical for the activation of NF-κB also was inhibited by VK2 (Fig 4B). The pan-PKC inhibitor Ro-31-8425 inhibited the TPA-induced phosphorylation of both PKD1 and IκBα, while the PKCα inhibitor Gö6976 showed no effect on the phosphorylation of both proteins (Fig. 4B). These results suggest that PKCδ or/and PKCε is/are involved in the phosphorylation of both PKD1 and IκBα. To confirm this notion, siRNA-mediated knockdown of PKC isoforms was employed. As shown in Fig. 4C, knockdown of PKCδ

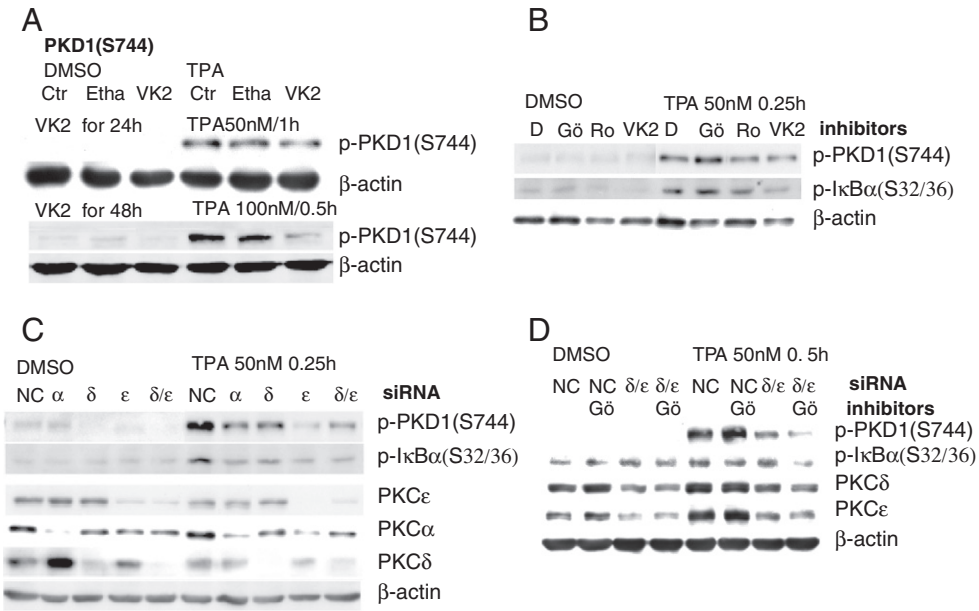

Fig. 4. VK2 suppressed the activation of PKD1, and PKCs are involved in the activation of PKD1 and IκBα in Huh7 cells. Activation of PKD1 and IκBα was assayed by Western blotting analysis using specific antibodies for p-PKD1 (S744) and p-IκBα (S32/36). (A) VK2 suppressed the activation of PKD1 in Huh7 cells. Cells were cultured in the presence of 10^{-4} M VK2 or the VK2 solvent ethanol as a control for 24 or 48 h and treated with or without TPA as indicated in the figure. (B) VK2 and pan-PKC inhibitor suppressed the phosphorylation of PKD1 and IκBα. Huh7 cells were cultured in the presence of the PKCα inhibitor Gö6976 (10 nM), pan-PKC inhibitor Ro-31-8425 (100 nM), VK2 (10^{-4} M) and DMSO as a control for 24 h. The cells were subjected to Western blot analysis after treatment with or without TPA. (C) Knockdown of PKCs inhibited the phosphorylation of PKD1 and IκBa. Huh7 cells cultured in 3.5 cm dishes were transfected with 20 nM siRNA of negative control (NC), PKCα (10 nM+10 nM NC), PKCδ (10 nM+10 nM NC), PKCε (10 nM+10 nM NC) and both PKCδ and PKCε (10 nM of each) as described in Materials and Methods. After culturing for 24 h, cells were treated with or without TPA and subjected to Western blot analysis. (D) Combination of knockdown of PKCδ and ε and PKCα inhibitor Gö6976 suppressed the activation of IκBα and PKD1 significantly. Huh7 cells were transfected with siRNA of negative control (NC), and both PKCδ and PKCε (10 nM of each) as described in panel (C). After 24 h, cells were treated with or without PKCα inhibitor Gö6976 (10 nM) for 1h and then induced with or without TPA for 0.5 h. NC, negative siRNA; α, PKCα siRNA; δ, PKCδ siRNA; ε, PKCε siRNA; Gö, PKCα inhibitor Gö6976; Ro, pan-PKC inhibitor Ro-31-8425. VK2, 10^{-4} M VK2.

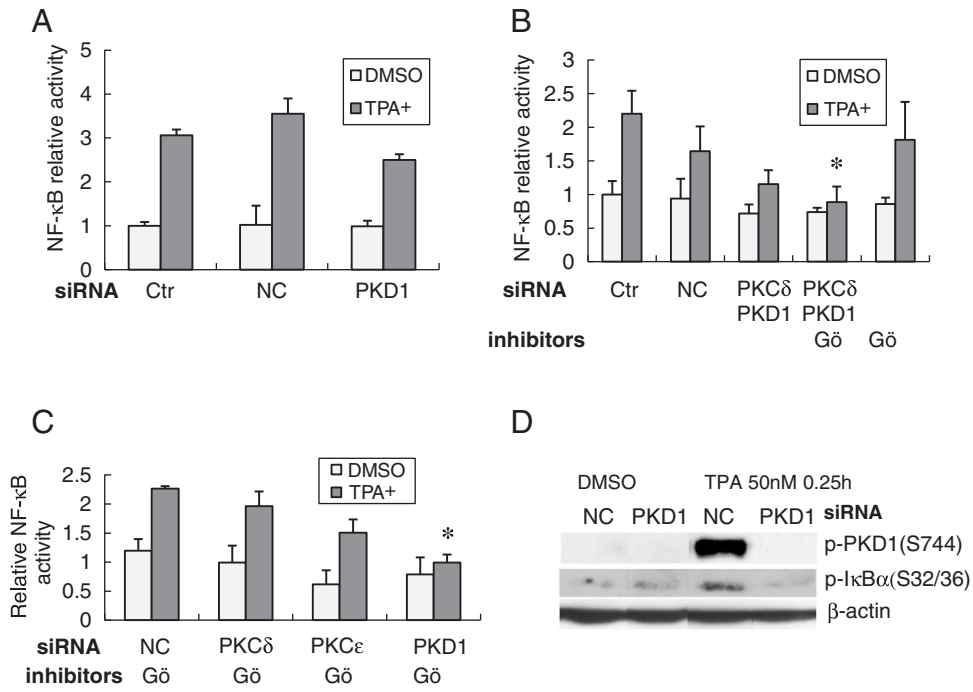

Fig. 5. PKD1 is involved in the TPA-induced activation of NF-κB. (A) Effect of PKD1 knockdown on NF-κB activity. Huh7 NF-κB reporter gene transfectant cells in 48-well plates were transfected with siRNA of negative control (NC) or PKD1. After culturing for 24 h, cells were treated with or without TPA (50 nM) for 3 h and subjected to luciferase assay. (B) Combination of knockdown of PKCδ, PKD1 and PKCα inhibitor Gö6976 suppressed the NF-κB activity to a similar level as pan-PKC inhibitor did. Huh7 NF-κB reporter gene transfectants cultured in 48-well plates were transfected with siRNA of negative control (NC) or PKCδ+PKD1 (10 nM of each). After culturing for 24 h, cells were treated with or without PKCα inhibitor Gö6976 (10 nM) for 24 h and subjected to luciferase assay after treatment with or without TPA. (C) Combination of knockdown of PKD1 and Gö6976 suppressed the NF-κB activity to a similar level as pan-PKC inhibitor did. Huh7 NF-κB reporter gene transfectants cultured in 48-well plates were transfected with 10 nM siRNA of negative control (NC), PKCα, PKCδ, PKCε and PKD1 and cultured for 24 h. Then, the cells were treated with or without PKCα inhibitor Gö6976 (10 nM) for 24 h and subjected to luciferase assay after with or without TPA induction. (D) At 24 h after transfection with 20 nM of PKD1 specific siRNA, phosphorylation of IκBα was suppressed effectively. Huh7 cells in 3.5-cm dishes were transfected with 20 nM siRNA of negative control (NC) or PKD1. After culturing for 24 h, cells were treated with or without TPA and subjected to Western blotting analysis with p-PKD1(S744) and p-IκBα (S32/36) specific antibodies. Bars, S.D.; *P<.05 (Student's t test); Ctr, no treatments; NC, negative siRNA; Gö, PKCα inhibitor Gö6976; Ro, pan-PKC inhibitor Ro-31-8425.

or/and PKCε decreased the phosphorylation of PKD1, but the knockdown of PKCα did not at the basal level. On TPA induction, however, the phosphorylation of both PKD1 and IκBα was decreased in all cases of knockdown of PKCα, PKCδ, PKCε and δ/ε compared with the control siRNA (Fig. 4C). On the knockdown of both PKCδ and ε, the phosphorylation levels of both PKD1 and IκBα were decreased compared to the levels of individually knocked down cells (Fig. 4C and D). When the cells simultaneously knocked down with PKCδ and ε siRNAs were treated with the PKCα inhibitor Gö6976, the TPA-induced phosphorylation of PKD1 and IκBα was further decreased (Fig. 4D). These results indicate that PKD1 signaling pathway might contribute to the IκBα phosphorylation that subsequently activates NF-κB.

To confirm the role of PKD1, siRNA-mediated knockdown of PKD1 was employed. The NF-κB reporter gene transfectants of Huh7 were transfected with PKD1 specific siRNA and subjected to luciferase assay. Knockdown of PKD1 only slightly decreased the TPA-induced NF-κB activity compared with the activity of negative control (Fig. 5A), while the TPA-induced NF-κB activity in PKD1 knockdown cells was significantly decreased by the treatment with the PKCα inhibitor Gö6976 (Fig. 5C and D). Furthermore, PKD1 knockdown decreased TPA-induced phosphorylation of IκBα (Fig. 5D). Altogether, the results indicate that PKD1 is involved in the TPA-induced activation of NF-κB, and all of the PKC isoforms, α, δ and ε, more or less contribute to the activation of PKD1.

4. Discussion

VKs have been revealed to have diverse effects, such as anticancer potential and antiosteoporotic effects, in addition to their essential roles in blood coagulation factors production [1,2]. Epidemiological studies have suggested a protective role for VKs against the development of atherosclerotic diseases [27]. Several mechanisms of VK2 action with regard to these effects have been reported. VKs have been shown to bind nuclear steroid xenobiotic receptor SXR (PXR) in bone-derived cells and to suppress osteoclastogenesis [28]. VKs also suppress the proliferation of several types of cancer cells [5–7]. In HCC cells, it has been reported that the activation of protein kinase A is involved in the growth suppression by VK [10,29]. We have revealed that VK2 inhibits the growth of HCC cells by suppressing cyclin D1 expression through the inhibition of NF-κB activation by suppressing IKK activity [13]. The suppression of NF-κB activation by VK was also reported in lipopolysaccharide-mediated macrophage activation [30] and in the VK-mediated suppression of osteoclastogenesis of bone cells through the RANK/RANKL pathway [31], suggesting that the suppression of NF-κB activation might play important roles in the activities of VK2.

NF-κB is a well-characterized transcription factor involved in the wide variety of important cellular functions such as the immune response, cell survival, apoptosis and carcinogenesis. Two distinct pathways have been described to activate NF-κB, and a number of factors are involved in the regulation of NF-κB activity (reviewed in Refs. [32,33]). PKCs are involved in the activation of NF-κB, and each isoform plays a different role in the regulation of NF-κB activation in various diseases [19,20,34,35]. Since VK2 inhibited the TPA-mediated activation of NF-κB, we hypothesized that VK2 might regulate PKC activation or enzyme activity. In the present study, we showed that all of the phospholipid-dependent PKCs, PKCα, δ and ε, which are expressed in Huh7 HCC cells participate to some degree in the activation of NF-κB. The simultaneous inhibition of three

PKCs, α, δ and ε, but not just two of them and treatment with a pan-PKC inhibitor resulted in the complete inhibition of TPA-induced NF-κB activation.

Interestingly, PKCε knockdown almost completely inhibited the TPA-induced phosphorylation of PKD1, a substrate of PKCε, indicating that PKCε stimulates NF-κB activity via PKD1 [35]. Knockdown of PKCα and PKCδ by siRNAs also inhibited the TPA-induced phosphorylation of PKD1, although the inhibition was partial (Fig. 4C), suggesting the involvement of PKCα and PKCδ in the phosphorylation of PKD1. The mechanism by which PKCα and PKCδ interact with PKD1 in Huh7 cells is not clear currently, but the regulation of PKD1 by PKCα [36] and PKCδ [26] was reported. The activity of PKCα and the phosphorylation of PKCε were inhibited by VK2 treatment, but PKCδ was not. Knockdown of PKCα decreased PKD1 phosphorylation, while the PKCα specific inhibitor showed no effect on the phosphorylation of PKD1. This might be due to the difference in mode and/or magnitude of inhibition between siRNA-mediated knockdown and pharmacological inhibition. Alternatively, the concentration of pharmacological inhibitor (10 nM of Gö6976) might not be enough to inhibit the phosphorylation of PKD1 because we used relatively low concentration of inhibitors to avoid the emergence of nonspecific kinase inhibitory action. PKD1 knockdown could not suppress completely the NF-κB activity to the same level as VK2 did, while the PKD1 knockdown with PKCα inhibitors reduced the NF-κB activity to the levels achieved by VK2. The results suggest that at least both PKCα- and PKCε/PKD1-mediated pathways are involved in the NF-κB activation by TPA, and the simultaneous suppression of both pathways might be required for enough suppression of TPA-induced NF-κB activation.

PKD1 was first identified as a PKC effector and was previously classified as PKCμ. However, PKD now constitutes a novel family of serine/threonine kinases and is classified as a subfamily of the calcium/calmodulin-dependent kinase superfamily. Recently, PKD has been shown to be dysregulated in several diseases, and it is regarded as a key regulator of diverse cellular functions such as cell growth, apoptosis, invasion and angiogenesis [37,38]. We have previously reported that VK2 suppressed the expression of MMPs, which are involved in the invasion and metastasis of cancer cells [15], in human HCC cell lines through the inhibition of NF-κB and/or the MAP kinase pathway [14]. Furthermore, VK has been shown to regulate angiogenesis through the modulation of VEGF [11]. PKD1 is also known to be involved in MMP expression [39,40] and VEGF-induced angiogenesis [36]. Endothelial cell proliferation, migration and angiogenesis by VEGF require PKD activity [41]. Therefore, VK2-mediated suppression of MMP expression and/or angiogenesis might involve the modulation of PKD activation.

The expression and distribution of PKCs are cell type and isoform specific. The PKCα, δ and ζ isoforms are found in all cells. The γ isoform is found only in neuronal cells, whereas the η and τ isoforms are predominantly expressed in epithelial and immune cells. The η, ε and λ isoforms are located in cells from various tissues. PKCs serve as the receptor for tumor-promoting phorbol esters, such as TPA, and the sustained activation of PKCs is associated with tumor promotion. Altered expression of PKCs has been linked with various types of malignancies, including HCC [42–44]. The activity of PKCs is regulated through three distinct conserved Ser/Thr phosphorylation sites: the A-loop, TM and HM [45,46]. In earlier literature, it was generally believed that phosphorylation at the A-loop was mediated by phosphoinositide-dependent kinase-1 (PDK1), which has been shown to be the upstream kinase for several members of the AGC family of kinases. Once phosphorylated at the A-loop, autophosphorylation at the TM and the HM occurred under the required conditions [47]. However, recent studies indicated that conventional PKCs can be phosphorylated at the TM and HM by the mammalian target of rapamycin complex 2 [48,49]. More recently, PKCδ, one of the novel PKCs, was found to be transphosphorylated at the A-loop by another novel PKC, PKCε, in place of PDK1 [50].

In conclusion, our data suggested that there are at least two pathways in PKC-mediated NF-κB activation in Huh7 cells, PKCα-NF-κB and PKCs-PKD1-NF-κB signaling pathways, and that VK2 might suppress the NF-κB activation via the inhibition of PKCα and PKCs (mainly PKCε)-PKD1 pathway. VKs have been shown to influence a wide variety of disease conditions, including bone health, cardiovascular diseases and neoplastic diseases, although the mechanisms by which VKs exert these diverse effects require further studies. NF-κB and PKCs/PKD are critical regulators for maintaining biological homeostasis; therefore, the understanding of the action of VKs involving NF-κB and PKCs/PKD will provide further information that can be useful for the prevention of a wide variety of diseases.

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