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ORIGINAL ARTICLE

Extracellular signal-regulated kinase phosphorylation due to menadione-induced arylation mediates growth inhibition of pancreas cancer cells

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Abstract
Background Cytotoxicity of Vitamin K3 (VK3) is indicated to have the same mechanism with oxidative stress (H_2O_2). In the present study, we analyzed the differences and/or similarities in the cellular responses to oxidative stress and VK3 to clarify the mechanism of growth inhibition.
Methods Cell viability was determined by a test method with 3-[4, 5-dimethyl-thiazol]-2, 5-dephenyl tetrazolium bromide (MTT). Expressions of cellular proteins were evaluated by Western blot analysis.
Results The IC50 was calculated to be 47.3 ± 4.1 μM for VK3 and 2.2 ± 1.2 μM for H_2O_2. By Western blot analysis, VK3 or H_2O_2 was shown to induce rapid phosphorylation of extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinases (JNKs). H_2O_2-induced phosphorylation of ERK and JNK was almost complete inhibited by more than 100-μM genistein. VK3-induced JNK phosphorylation was blocked by 100-μM genistein, but ERK phosphorylation was not inhibited completely even if 400-μM genistein was used. H_2O_2-induced inhibition of cell proliferation was completely blocked by 400-μM genistein, but the VK3 effect was reduced 72.8 ± 5.4% by the same concentration of genistein. H_2O_2-induced JNK phosphorylation and ERK phosphorylation were inhibited by staurosporine, protein kinase C (PKC) inhibitor. VK3-induced JNK phosphorylation was also blocked, but ERK phosphorylation was not affected. Staurosporine had no effect on VK3- or H_2O_2-induced growth inhibition. Treatment with a non-thiol antioxidant agent, catalase, completely abrogated H_2O_2-induced JNK and ERK phosphorylation, but a thiol antioxidant, L-cystein, had no effect on phosphorylation of them. The VK3-induced JNK phosphorylation was inhibited by catalase, but not L-cystein. But ERK phosphorylation was not inhibited by catalase and was abrogated completely by the thiol antioxidant. Catalase, but not L-cystein, blocked H_2O_2-induced growth inhibition, and L-cystein, but not catalase, blocked VK3-induced effects on cell proliferation completely.
Conclusion VK3-induced ERK phosphorylation occurs by a different mechanism from oxidative stress, and it might have an important role to induce growth inhibition.

Keywords Menadione (vitamin K3) · Oxidative stress · Extracellular signal-regulated kinase (ERK)

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Introduction

Oxidative stress has been defined as elevated levels of reactive oxygen species (ROS) and impaired antioxidant defense system function [1]. ROS is generated in aerobic organisms during biochemical processes, as well as in response to various endogenous or exogenous stimuli. Although cells contain some antioxidant defense systems, when ROS production is increased, the antioxidant capacity is overwhelmed, resulting in oxidative stress, which occurs under several pathologic conditions [2, 3]. The toxic effects of oxidative stress depend upon the degree of stress and/or the type of cell. For example in hepatoma cells, low-ROS concentrations have been shown to increase antioxidant enzyme activity and promote cell proliferation, whereas, high-ROS concentrations inhibit cell growth [4]. Cancer

cells typically exhibit poor antioxidant status [5], thus raising the possibility of death of cancer cells through oxidative stress. Under these conditions, ROS has been found to activate molecules in various cellular signaling pathways [6]. Among these responses, activation of the mitogen-activated protein kinases (MAPKs) plays an important role in initiating oxidative damage-induced responses. C-Jun N-terminal kinases (JNKs) and p38 MAPK are activated by environmental stress and are closely related to cell death, whereas extracellular signal-regulated kinase (ERK) 1/2 is activated by growth factors and has an effect to prevent cell death. One of the most important mechanisms of MAPK phosphorylation in response to oxidative stress is induced by oxidative inhibition of the specific phosphatases [7]. Recent studies suggested that ROS may act as second messengers for transcription activation, apoptosis, cell growths, and chemotaxis [6]. The mechanisms by which ROS alter signal transduction and gene expression have been described for cell proliferation-induced mild oxidative stress, but the mechanism by which severe stress inhibits cell growth is still poorly understood [8].

Menadione (Vitamin K3, VK3) was shown to raise superoxide anion levels upon intracellular metabolism as ROS precursors [9], resulting in inhibiting cancer cell growth [10]. Because H_2O_2 can permeate the cell membrane, extracellular H_2O_2 activates signaling cascades even in the absence of a receptor ligand [11]. Similarly, extracellular H_2O_2 can have toxic effects not only on the cell membrane but also on intracellular structures. Because catalase is not present in the cellular environment the half-life of H_2O_2 outside cells is relatively long, and signaling as well as toxic effects are favored. Therefore, cytotoxicity of VK3 is caused by oxidative stress (H_2O_2) that leads to the generation of ROS. This oxidative stress is abolished by the addition of catalase, the enzyme that destroys H_2O_2 [12]. In the present study, we analyzed the differences and/or similarities in the cellular responses to oxidative stress and VK3 to clarify the mechanism of growth inhibition by ROS and VK3. The possible utility of VK3 for cancer therapy is discussed.

Materials and methods

Cell lines

The rat pancreatic cancer cell line ARIP was grown in F12K media (MP Biomedicals Inc., Costa Mesa, CA, USA). The media was supplemented with 10% fetal calf serum, penicillin, streptomycin, L-glutamine, and fungizone. For various tests, cells were harvested after trypsin-EDTA treatment, washed with Dulbecco's PBS, and re-suspended in serum-less media for 24 h.

Determination of cell viability

Cell viability was determined by a test method with 3-[4, 5-dimethylthiazol]-2, 5-dephenyl tetrazolium bromide (MTT). MTT (5 mg/ml) was dissolved in PBS, and the solution was stored at 2–8°C for frequent use after filtration though a 0.2 μm filter. To determine the effects of VK3 or H_2O_2 on cell growth inhibition, the medium was discarded, and the MTT solution was added and incubated for 3 h. At the end of the incubation period the MTT solution was removed and the dye crystals of the cells were dissolved by adding dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm in a spectrophotometer and the results were expressed as a percentage of the absorbance of the samples in comparison to control, as described previously [7].

Western blot analysis

Cell lysate was isolated and homogenized in radioimmunoprecipitation assay (RIPA) buffer [10 mmol/l Tris–HCl (pH 7.5) 150 mmol/l NaCl; 5 mmol/l EDTA; 0.5% SDS; 1.0% NP40; and 1.0% sodium deoxycholate-containing protease inhibitors] as described previously [7, 13]. The homogenate was incubated on ice for 30 min and centrifuged for 15 min at 14,000 rpm at 4°C. The supernatant was recovered and kept at −80°C. Protein determination assays were performed with the Bradford assay (Bio-Rad, Hercules, CA, USA), and equal amounts of cell tissue lysate (40 μg for each lane) were mixed with SDS-PAGE sample buffer and boiled for 5 min before loading onto a discontinuous SDS-PAGE gel. After electrophoresis, the gel was incubated in 3-(cyclohexylamino) propanesulfonic acid (CAPS) buffer [10 mmol/l 3-cyclohexylamino-1-propane-sulfonic acid (pH 11.0) 10% methanol] for 5 min, and proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories) in CAPS buffer. The blot was then blocked by incubating in 5% non-fat dried milk/TBS-Tween for 1 h at room temperature and incubated for 1 h with first antibody for phosphor-tyrosine (Cell Signaling, Beverly, MA, USA; 1:2,000), phospho-extracellular signal-regulated kinase (phospho-ERK) (Calbiochem, LaJolla, CA, USA; 1:1,000), phospho-c-Jun NH2-terminal kinase (phospho-JNK) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1,000) in 5% non-fat dried milk/TBS-Tween at room temperature. The membrane was washed three times for 20 min in TBS-Tween and additionally incubated with a secondary antibody mouse (Sigma, St. Louis, MO, USA; 1:1,000) for phosphor-JNK and phosphor-tyrosine and rabbit (Sigma; 1:500) for phosphor-ERK immunoglobulin conjugated with horseradish peroxidase in 5% non-fat dried milk/TBS-Tween for 1 h at room temperature. After washing three times for 30 min in TBS-Tween at room temperature, the signal was detected by using enhanced

chemiluminescence (Perkin-Elmer Life Science, Boston, MA, USA). Loading of protein was normalized by reprobing with a β-actin polyclonal antibody.

Statistical analysis

At least four independent determinations of each parameter were compared to control using Student's *t* test. Differences were considered significant when $P < 0.05$ was obtained.

Results

To determine the optimal concentrations of VK3 or H_2O_2 to inhibit cell growth in the ARIP pancreas cancer cell line, cells were treated with various concentrations of VK3 or H_2O_2 for 24 h. The IC50 was calculated to be 47.3 ± 4.1 μM for VK3 and 2.2 ± 1.2 μM for H_2O_2 (Fig. 1). Vehicle (ethanol) for VK3 alone had no effect on cell proliferation. VK3 concentrations of 100 μM and H_2O_2 concentrations of 5.0 μM were used in subsequent experiments. By Western blot analysis, VK3 or H_2O_2 was shown to induce rapid phosphorylation of tyrosine, ERK 1/2, and JNK for 30 min after application (data not shown). ERK and JNK were phosphorylated in response to treatment with 5.0 μM H_2O_2 but were diminished after 3 h. In contrast, phosphorylation of ERK and JNK was maintained over 6 h, and, for ERK, lasted at least 12 h (data not shown). Phosphorylation of another MAPK family member, p38, was not detected in response to treatment with these agents. As shown in Fig. 2, H_2O_2-induced phosphorylation of ERK, JNK, and tyrosine was almost completely inhibited by more than 100-μM genistein. VK3-induced tyrosine phosphorylation and JNK phosphorylation were blocked by 200- and 100-μM genistein, respectively, but ERK phosphorylation was not inhibited completely even if 400-μM genistein was used. H_2O_2-induced inhibition of cell proliferation was completely blocked by 400-μM genistein, but the VK3 effect was reduced $72.8 \pm 5.4\%$ by the same concentration of genistein (data not shown).

Study of protein kinase C (PKC) phosphorylation was then performed. PKC was phosphorylated by100 μM VK3 and 5.0 μM H_2O_2, but it was blocked by more than 50 μM staurosporine (data not shown). H_2O_2-induced JNK phosphorylation and ERK phosphorylation were inhibited by more than 50 μM staurosporine. VK3-induced JNK phosphorylation was also blocked by staurosporine, but ERK phosphorylation was not affected by staurosporine (Fig. 3). Staurosporine had no effect on VK3- or H_2O_2-induced growth inhibition (data not shown).

The effects of antioxidants on signal transduction pathways were studied. Treatment with a non-thiol antioxidant agent, catalase, completely abrogated H_2O_2-induced JNK/ERK phosphorylation and growth inhibition, but a thiol antioxidant, L-cystein, had no effect on them (Fig. 4). In contrast, antioxidants have different effects on VK3-induced cellular responses (Fig. 5). JNK phosphorylation was inhibited by catalase, but not L-cystein. But ERK phosphorylation was not inhibited by catalase and was abrogated completely by thiol antioxidant (0.2 mM). And L-cystein (over 0.2 mM), but not catalase, blocked VK3-induced effects on cell proliferation completely.

Discussion

To date, three major signal transduction pathways have been implicated in the regulation of the antioxidant response. They are the MAPK, phosphatidylinositol 3-kinase, and PKC pathways [14, 15]. The MAPKs are involved in essential signal transduction cascades that regulate cell growth, differentiation, apoptosis, and transformation [16]. PKCs are a large family of serine/threonine protein kinases, and their activation can activate various intracellular signal transduction systems [17]. Thus, activation of the PKC-MAPK pathway is considered one of the most important

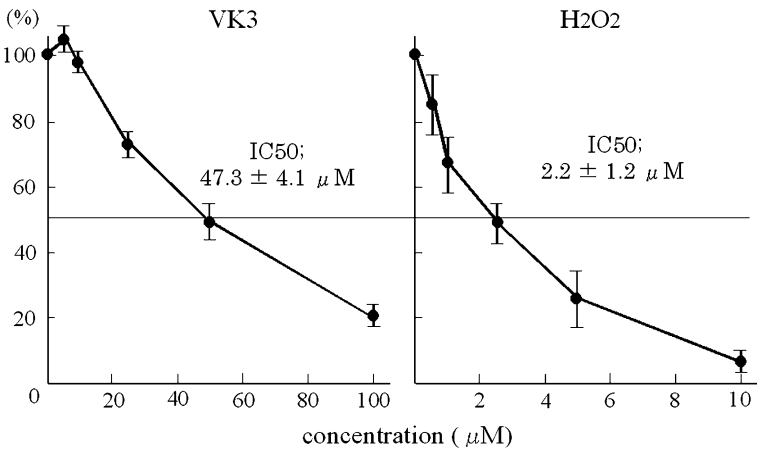

Fig. 1 Growth inhibitory effect of vitamin K3 (VK3) and H_2O_2. The ARIP pancreas cancer cells were treated with various concentrations of VK3 or H_2O_2 for 24 h. Cell viability was calculated by standard MTT assay, as described in "Materials and methods" section. The IC50 was calculated to be 47.3 ± 4.1 μM for VK3 and 2.2 ± 1.2 μM for H_2O_2

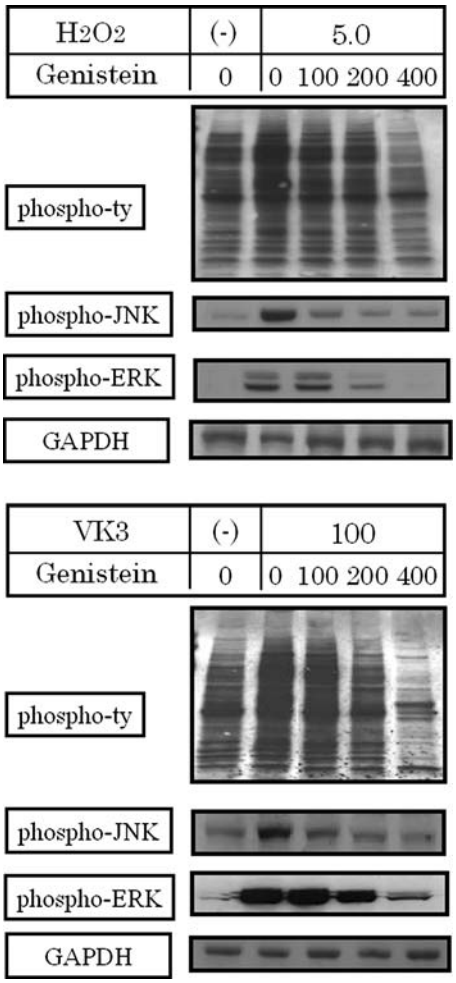

Fig. 2 The effect of genisten for VK3 or H_2O_2-induced phosphorylation of cellular proteins. VK3 or H_2O_2 induced phosphorylation of tyrosine, ERK 1/2, and JNK. H_2O_2-induced phosphorylation of ERK, JNK, and tyrosine was almost complete inhibited by more than 100-μM genistein. VK3-induced tyrosine phosphorylation and JNK phosphorylation were blocked by 200- and 100-μM genistein, respectively, but ERK phosphorylation was not inhibited completely even if 400-μM genistein was used. The expression of cellular proteins was evaluated by Western blot, as described in "Materials and methods" section

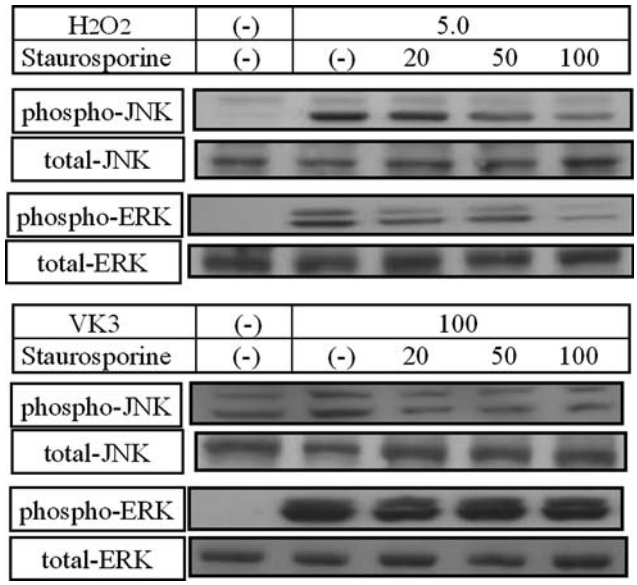

Fig. 3 The effect of protein kinase C inhibitor for VK3 or H_2O_2-induced phosphorylation of cellular proteins. H_2O_2-induced JNK phosphorylation and ERK phosphorylation were inhibited by more than 50 μM staurosporine. VK3-induced JNK phosphorylation was also blocked by staurosporine, but ERK phosphorylation was not affected by staurosporine

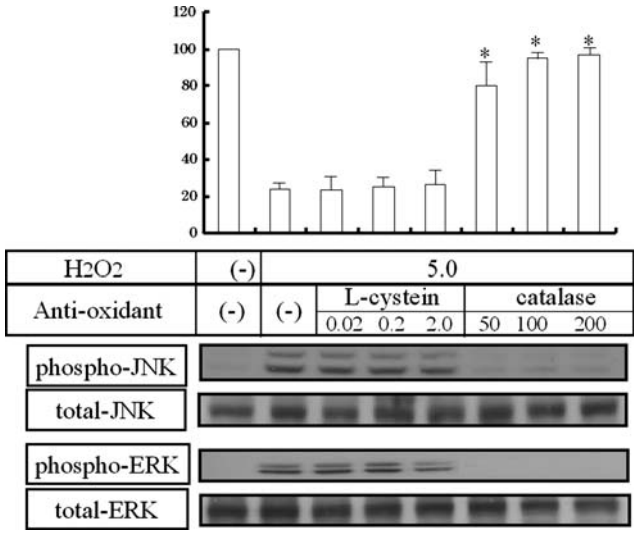

Fig. 4 The effect of antioxidant for H_2O_2-induced phosphorylation of cellular proteins and growth inhibition. Treatment with a non-thiol antioxidant agent, catalase, completely abrogated H_2O_2-induced JNK/ERK phosphorylation and growth inhibition, but a thiol antioxidant, L-cystein, had no effect on them (*$P < 0.05$ compared to without antioxidant)

molecular mechanisms for control of oxidative stress [18]. Indeed, overexpression of PKC is reported to activate ERK [19–21]. In the present study, oxidative stress-induced phosphorylation of ERK and JNK was completely antagonized by PKC inhibitor, suggesting that PKC acts upstream of the MAPK pathway. However, the PKC inhibitor blocked VK3-induced JNK phosphorylation, but not ERK, indicating that VK3-mediated phosphorylation of ERK occurs independent of PKC. In ovarian cancer cells, activation of ERK 1/2 through a PKC-dependent pathway is thought to be essential for the anti-proliferative effects of gonadotropin-releasing hormone [22]. In contrast, in pancreatic cancer cell lines, PKC-independent ERK activation has some connection with the growth inhibitory effect of VK3. VK3-induced growth inhibition and ERK phosphorylation may have different mechanisms from ROS-related actions.

Protein tyrosine phosphorylation is regulated by the balance of the activities of tyrosine phosphatases (PTPases) and protein tyrosine kinases (PTKs). Because oxidative stress inhibits the activity of PTPases [23], H_2O_2 inhibits

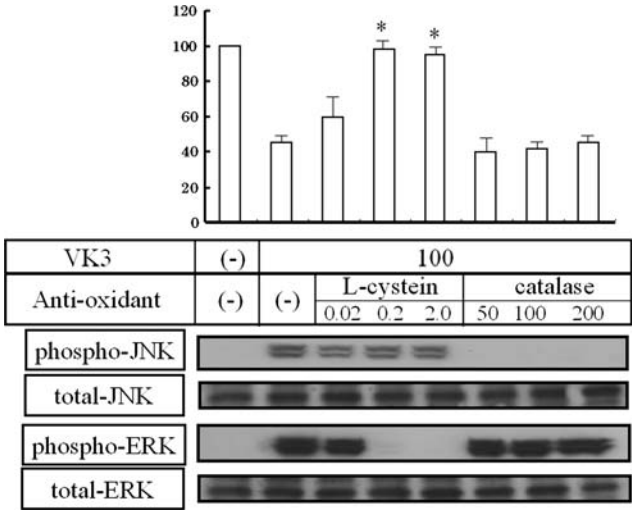

Fig. 5 The effect of antioxidant for VK3-induced phosphorylation of cellular proteins and growth inhibition. VK3-induced JNK phosphorylation was inhibited by catalase. But ERK phosphorylation was not inhibited by catalase and was abrogated completely by thiol antioxidant. And L-cystein (over 0.2 mM), but not catalase, blocked VK3-induced effects on cell proliferation completely (*$P < 0.05$ compared to without antioxidant)

PTPase of PKC, resulting in activation of PKC, then the upstream stimulation of PKC was suggested to induce phosphorylation of ERK and JNK. In addition from the present study, VK3-induced JNK phosphorylation was also blocked by PKC inhibitor. And genistein can antagonize VK3-induced JNK phosphorylation by the similar concentration to block protein tyrosine phosphorylation (Fig. 2). In contrast, VK3-induced ERK phosphorylation, even if PKC and/or whole tyrosine phosphorylation inhibitors (staurosporine and genistein, respectively) were present. It was recently reported that quinone sulfides arylate thiols by an additional elimination mechanism [24–26], which is a different mechanism from oxidative stress. VK3, which is a quinone sulfide, is likely bind to critical cysteine residues in the active site of PTPases. Because ERKs are dually phosphorylated on tyrosines and threonines, ERK may be potential targets of such an arylation system. In the present study, the results of the antioxidant experiments (Fig. 4) suggested that VK3-induced ERK phosphorylation may be due directly to arylation-induced inhibition of PTPase. Thus, VK3 was indicated to induce both growth inhibition and ERK phosphorylation due to arylating action. Among members of the MAPK pathway, JNK and p38 are believed to have pro-apoptotic roles, whereas ERK has functions to block cell death [27]. In contrast, recent studies have suggested that the ERK pathway promotes apoptosis [28–30]. Transient or prolonged expression of phosphorylated ERK was found to determine the cellular response for growth stimulation or inhibition [28]. In the present pancreas cancer cell line, ERK phosphorylation was sustained over 12 h after VK3 application. Therefore, as an anti-cancer drug, VK3 may have an additional effect, inhibition of PTPases by arylation, that contributes to oxidative stress in cancer cells. Further studies to clarify the mechanisms of VK3-induced growth inhibition are needed.

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