Inhibition of PTEN and activation of Akt by menadione

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Abbreviations: CHO-IR cells, Chinese hamster ovary cells expressing insulin receptors; IRβ, the β subunit of insulin receptor; PI 3-kinase, phosphoinositide 3-kinase; pNPP, p-nitrophenyl phosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10

Abstract

Menadione (vitamin K_3) has been shown to activate Erk in several cell lines. This effect has been shown to be due to the activation of EGF receptors (EGFR) as a result of inhibition of some protein tyrosine phosphatases. In the present study, we examined the effects of menadione on Akt in Chinese hamster ovary cells. The phosphorylation of Akt by menadione was not inhibited by AG1478, an inhibitor of EGFR. Menadione inhibited the lipid phosphatase activity of PTEN in a cell-free system. In an intact cell system, menadione inhibited the effect of transfected PTEN on Akt. Thus, one mechanism of its action was considered the accelerated activation of Akt through inhibition of PTEN. This was not the sole mechanism responsible for the EGFR-independent activation of Akt, because menadione attenuated the rate of Akt dephosphorylation even in PTEN-null PC3 cells. The decelerated inactivation of Akt, probably through inhibition of some tyrosine phosphatases, was considered another mechanism of its action.

1. Introduction

Vitamin K is a fat-soluble vitamin essential for the post-translational modification of proteins, including the coagulation factors II (prothrombin), VII, IX, X, and proteins C, S, and Z [1]. Two naturally occurring forms of vitamin K, phylloquinone (vitamin K_1) and menaquinone (vitamin K_2), act as coenzymes for γ-glutamyl carboxylase, which produces γ-carboxyglutamic acid (Gla) by carboxylating the side chain of glutamic acid [2]. Vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone) is a synthetic vitamin K congener that is not a co-factor for γ-glutamyl carboxylase. Menadione has been examined extensively for its inhibitory effect on the proliferation of tumor cells both in vivo and in vitro [3-6]. Similarly to various quinones, menadione can be reduced in cells to produce the semiquinone radical, which may impair the cellular constituents directly or indirectly through production of reactive oxygen species. The 3-position of menadione is also expected to react on the sulfhydryl group of glutathione by the Michael-type addition mechanism [7,8]. Thus, menadione produces oxidative stress on cells through its ability to undergo both redox cycling and conjugate formation. In support of the occurrence of oxidative stress, CHO cells showing resistance to menadione-induced cell death have been reported to exhibit increased concentrations of glutathione and cysteine [9]. On the other hand, some effects of naphthoquinone derivatives are considered to be independent of oxidative stress [10-12].

Menadione has been shown to activate the Erk cascade [13-15]. Abdelmohsen et al. suggested that this action of menadione may be separated from the oxidative stress

[12]. They showed that the prior treatment of cells with the antioxidant, N-acetyl cysteine, which prevents the effect of the redox cycler, 1,4-benzoquinone, did not attenuate the effect of menadione. They also showed that menadione had a minor effect on the cellular level of glutathione at the concentration at which it activates Erk. Recent studies have indicated that menadione induces phosphorylation and activation of Erk by activating EGF receptors (EGFR), because pharmacological inhibitors of the receptor tyrosine kinase attenuated menadione-induced activation of Erk [12,15,16]. This conclusion was confirmed by the results of EGFR desensitization [12].

Menadione has been reported to inhibit protein tyrosine phosphatases in cell-free systems [15-17]. An analog of menadione has been shown to be a potent inhibitor of Cdc25 with minor effects on other protein tyrosine phosphatases [18]. The basis of these inhibitory effects is proposed to be the direct interaction of the compounds with the cysteine residue that plays an essential role in the process of tyrosine dephosphorylation [17,19,20]. Based on these observations, menadione is proposed to perturb the balance between the phosphorylation and dephosphorylation of EGFR leading to activation of the Erk cascade [12,16]. The effect of menadione on the PI 3-kinase/Akt cascade has also been observed and thus attributed to the activation of EGFR in rat liver epithelial cells [12].

In the present study, we showed that menadione activates Akt in Chinese hamster ovary cells. This effect was dependent on PI 3-kinase, but was refractory to inhibition of EGFR/tyrosine kinase. Our results indicated that one target of menadione is PTEN (phosphatase and tensin homolog deleted on chromosome 10), which

hydrolyzes and inactivates the products of PI 3-kinase [21,22]. We also observed that menadione inhibits the dephosphorylation of both serine and threonine residues of Akt in intact cells. These effects of menadione were considered the basis of the EGFR-independent activation of Akt.

2. Materials and methods

2.1. Materials

Menadione (2-methyl-1,4-naphthoquinone, vitamin K_3), insulin, bovine serum albumin (fatty acid-free), Ni-CAM HC resin, and anti-myc antibody (9E10) were purchased from Sigma (St. Louis, MO). 1,4-Benzoquinone was from Tokyo Kasei (Tokyo, Japan). Wortmannin, LY294002, and AG1478 were from Kyowa Medex (Tokyo, Japan), Cayman Chemical (Ann Arbor, MI), and Merck (Darmstadt, Germany), respectively. [γ-^{32}P]ATP was obtained from Perkin-Elmer (Norwalk, CT). Antibodies against pThr-308 and pSer-473 of Akt were from Cell Signaling Tech (Beverly, MA). Polyclonal anti-Akt antibody, polyclonal anti-IRβ antibody, and monoclonal anti-phosphotyrosine antibody (PY99) were from Santa Cruz (Santa Cruz, CA). Anti-mouse IgG agarose was from American Qualex Antibodies (San Clemente, CA). pGEX-6P-1, precision protease, and PD-10 column were from GE Healthcare (Buckinghamshire, UK). pQE-30 expression vector was from Qiagen (Hilden, Germany).

2.2. Recombinant proteins

cDNA encoding PTEN (GenBank accession number NM000314) was provided by Dr. T. Maehama (National Institute of Infectious Diseases, Tokyo, Japan). cDNAs encoding PTP1B, SHP-1, SHP-2, Cdc25A, and VHR (GenBank accession numbers NM002827, NM002831, NM003834, NM001789, and NM004090, respectively) were obtained by polymerase chain reaction with appropriate cDNA libraries and primers possessing additional nucleotide sequences convenient for subcloning. The cDNA constructs were subcloned into the expression vector pGEX-6P-1, pET28a, or pQE-30. GST (glutathione S-transferase)-fused or 6xHis-tagged proteins were expressed in *Escherichia coli* and adsorbed onto glutathione Sepharose 4B or Ni-CAM HC resin, respectively, in accordance with the manufacturer's instructions. The glutathione beads were incubated with precision protease, and the cleaved proteins were stored at –80°C in Tris-buffered saline containing 1 mM dithiothreitol (DTT). His-tagged proteins were eluted from the Ni-CAM beads with 250 mM imidazole. The eluate was applied to a PD-10 column to remove imidazole before storage at –80°C.

2.3. Cell lines

Chinese hamster ovary cells expressing insulin receptors (CHO-IR cells) were kind gifts from Dr. Y. Ebina (Ehime University, Matsuyama, Japan) and were cultured in F12-Ham's medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin. PTEN-null PC3 cells were obtained from Dr. T. Maehama (National Institute of Infectious Diseases, Tokyo, Japan) and maintained in

DMEM supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin.

2.4. Immunoblotting

The cells were lysed in lysis buffer consisting of 25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM NaF, 200 μM phenylmethylsulfonyl fluoride (PMSF), 20 μM (4-amidinophenyl)-methylsulfonyl fluoride (pAPMSF), 2 μM leupeptin, 2 μM pepstatin, and 1% Nonidet P-40. After centrifugation (15,000 rpm for 10 min), aliquots of the supernatant were mixed with SDS-PAGE sample buffer and boiled for 5 min. The peptides were separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking, the membranes were incubated with the indicated antibody, washed, and then incubated with horseradish peroxidase-conjugated second antibody. The second antibody was located using an enhanced chemiluminescence detection system (Perkin-Elmer, Norwalk, CT).

2.5. Lipid phosphatase activity

Recombinant PTEN (50 ng) was incubated in the presence or absence of menadione at 37°C for 5 min in 10 μl of buffer consisting of 0.1 M Tris-HCl (pH 8.0), 10 mM DTT, 0.25% octyl glucoside, and 50 μg/ml bovine serum albumin. The phosphatase reaction was started by addition of 10 μl of 0.2 mM diC_{16}-PtdIns-3,4,5-P_3 and was stopped after 5 min by addition of 20 μl of 0.1 M *N*-ethylmaleimide. After

centrifugation (15,000 rpm for 20 min), aliquots of 20 μl of the supernatant were mixed with 80 μl of GREEN Reagent (BIOMOL, Plymouth Meeting, PA). After development of the color for 15 min, absorbance at 620 nm was measured. The amount of phosphate released was quantified using standard solutions of inorganic phosphate.

2.6. PI 3-kinase activity

CHO-IR cells, cultured on six-well plates, were starved of serum for 15 h. After treatment with insulin and/or menadione, the cells were solubilized in 25 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, 30 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, 200 μM PMSF, 20 μM p-APMSF, 2 μM leupeptin, 2 μM pepstain, and 1% Nonidet P-40. After centrifugation at 15,000 rpm for 20 min, the supernatant was subjected to immunoprecipitation with anti-pTyr and anti-mouse IgG-conjugated agarose beads. The beads were washed twice with the same buffer, twice with 40 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 1 mM DTT, and once with Tris-HCl buffer containing 0.5 mM EGTA. The beads were suspended in 100 μl of Tris-HCl buffer containing 0.25 mM EGTA, 2.5 mM $MgCl_2$, 0.2 mM phosphatidylserine, 0.2 mM phosphatidylinositol, and 0.2 mM [γ-^{32}P]ATP (50 μCi/ml). The reaction was allowed to proceed at 30°C for 15 min and stopped by the addition of 20 μl of 8% $HClO_4$. To the mixture was added 450 μl of methanol-$CHCl_3$ (2:1), stirred vigorously, and then 150 μl of each of $CHCl_3$ and methanol were added. The organic phase was washed twice with $CHCl_3$-saturated solution containing 0.5 M NaCl and 1% $HClO_4$. After drying, the extract was spotted onto Silica Gel 60 plates (Merck,

Darmstadt, Germany), which was developed in $CHCl_3$/methanol/28% NH_4OH/H_2O (70:100:25:15). The radioactivity in the PtdIns-3-P spot was determined using Fuji BAS2000 analyzer (Fuji, Tokyo, Japan).

2.7. Dephosphorylation of IRβ

After CHO-IR cells were starved of serum for 15 h, the medium was replaced with incubation buffer consisting of 130 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES-NaOH (pH 7.4), and 0.1% (w/v) bovine serum albumin. The cells were incubated with or without 30 μM menadione for 15 min, and then for 4 min with the addition of 100 nM insulin. The cells were washed rapidly with acidified buffer (pH 6.0) to remove insulin and then incubated with or without 30 μM menadione before the reaction was stopped by adding 1 ml of ice-cold phosphate-buffered saline. The cells lysates were used for Western blotting analysis with anti-pTyr and anti-IRβ.

2.8. p-Nitrophenyl phosphate phosphatase activity

PTP1B (100 ng; 2 pmol), SHP1 (500 ng; 7.5 pmol), SHP2 (5 μg; 100 pmol), Cdc25A (50 μg; 850 pmol), or VHR (2 μg; 100 pmol) were incubated at 30°C for 10 min in 90 μl of reaction buffer containing various concentrations of menadione. The reaction buffer for the activities of PTP1B, SHP1, SHP2, and Cdc25A consisted of 100 mM sodium acetate (pH 5.5), 135 mM NaCl, 50 ng/μl of bovine serum albumin, and 2 mM DTT. The activity of VHR was assayed in buffer consisting of 25 mM

3-(*N*-morpholino)propanesulfonic acid (pH 6.0), 5 mM EDTA, and 1 mM DTT. The phosphatase reaction was started by addition of 10 μl of 50 mM p-nitrophenyl phosphate (pNPP) solution and stopped after 10 min by addition of 100 μl of 1 N NaOH. The amount of p-nitrophenol produced was determined from the absorbance at 405 nm. The pNPP phosphatase activity of PTEN was assayed in buffer consisting of 100 mM sodium acetate (pH 5.5), 135 mM NaCl, 50 ng/μl of bovine serum albumin, and 10 mM DTT. The reaction was allowed to proceed at 20°C for 15 hour before being stopped.

3. Results

3.1. Menadione activates Akt in CHO cells without activating EGF receptors

Treatment of CHO cells with menadione or insulin caused the phosphorylation of Akt on both the Ser-473 residue in the C-terminal hydrophobic motif and the Thr-308 residue in the activation loop (Fig. 1a). The effects were inhibited completely by the PI 3-kinase inhibitors, wortmannin and LY294002 (Fig. 1a). The oxidative stress may not play a major role in Akt activation because the potent redox cycler 1,4-benzoquinone (30 μM) did not cause marked activation of Akt (data not shown). In several cell lines, menadione has been shown to activate Erk by activating endogenous EGFR [12,15,16]. In contrast to these studies, a pharmacological inhibitor of EGFR (AG1478) showed no effect on menadione-induced Erk phosphorylation (Fig. 1b). The phosphorylation of Akt was again unaffected by AG1478 (Fig. 1b). These results indicated that EGFR does not function in CHO cells. In fact, treatment of the cells with EGF did not induce phosphorylation of Erk or Akt (Fig. 1b). In a control

experiment using PC12 cells, AG1478 inhibited the EGF-induced activation of both Erk and Akt (Fig. 1c).

3.2. Menadione does not affect PI 3-kinase activity

The activity of PI 3-kinase is known to increase when tyrosine-phosphorylated proteins bind to the SH2 domains of its regulatory subunit [23]. We have reported that a naphthoquinone derivative, shikonin, inhibits the ability of recombinant PTP1B to dephosphorylate both pNPP and the insulin receptor [24]. As menadione and its derivatives have been shown to inhibit some tyrosine phosphatases [15-17], one possible explanation for the EGFR-independent activation of Akt is the inhibited dephosphorylation and increased activity of insulin receptors. The experiment shown in Fig. 2 was performed to examine this possibility. IRβ in insulin-treated cells was in a highly phosphorylated state (Fig. 2a). The removal of insulin by washing the cells with acidified medium (pH 6.0) caused rapid dephosphorylation of IRβ. The rate of dephosphorylation was unchanged when the same experiment was performed in the presence of 30 μM menadione (Fig. 2b). These results indicated that the rate of IRβ dephosphorylation is unaffected by menadione. Another explanation for the action of menadione is the receptor-independent activation of cellular PI 3-kinase. However, we could not detect the presence of the active complex of PI 3-kinase in menadione-treated cells (Fig. 3). The insulin-induced activation of PI 3-kinase was again unaffected by menadione (Fig. 3). These results indicated that neither the activation nor inactivation mechanisms of PI 3-kinase were influenced by menadione.

3.3 Menadione inhibits PTEN and Cdc25A in cell-free systems

Akt in insulin-treated cells was in a highly phosphorylated state due to the accumulation of PtdIns-3,4,5-P_3. When the PI 3-kinase activity in the cells was abruptly blocked by addition of wortmannin, the phosphorylation decreased gradually and disappeared almost completely after 5 min (Fig. 4a). In cells treated with 30 μM menadione, the rate of dephosphorylation was markedly slowed (Fig. 4b). One possible explanation for the decreased Akt dephosphorylation is that menadione inhibited the dephosphorylation and inactivation of PtdIns-3,4,5-P_3. In the experiment shown in Fig. 4c, recombinant PTEN was incubated with 50 μM PtdIns-3,4,5-P_3 and the liberation of phosphate was determined by Malachite green assay. The results clearly show that the lipid phosphatase activity of PTEN is inhibited by menadione.

As PTEN possesses the signature motif HCXXGXXR present in enzymes with tyrosine phosphatase activity [22,25], we examined the effect of menadione on the pNPP (p-nitrophenyl phosphate) phosphatase activities of both PTEN and tyrosine phosphatases (Fig. 5). The recombinant enzymes were incubated with the indicated concentrations of menadione, and the phosphatase reaction was started by addition of 5 mM pNPP. The activity of PTEN was inhibited by menadione with IC_{50} value of 3 μM (Fig. 5a). The value was lower than that required to inhibit the lipid phosphatase activity (Fig. 4c). The difference may be due to the higher K_m value of pNPP as a substrate of PTEN (>20 mM). The activities of specific tyrosine phosphatases, PTP1B, SHP-1, and SHP-2, showed no or lower sensitivity to menadione (Fig. 5b). The activity of a

dual-specificity phosphatase, Cdc25A, was effectively inhibited by menadione (Fig. 5a) in agreement with the results of previous studies [17,26]. The inhibition under the experimental conditions (pH 5.5) showed a biphasic pattern; the activity halved at 1 μM menadione and abolished at 100 μM. When the experiment was performed at pH 8.0, the inhibition showed a monophasic pattern with IC_{50} value of 5 μM (data not shown). Another dual-specificity phosphatase, VHR, the active pocket of which is reported to be shallow and thus similar to PTEN [27], was not inhibited at all (Fig. 5a). Thus, menadione possesses some selectivity toward PTEN and Cdc25A relative to other protein tyrosine phosphatases. It has been reported that an analog of menadione (Cpd 5), which is expected to interact with the catalytic cysteine residues of the tyrosine phosphatases, inhibits both Cdc25A and VHR [18]. The difference in the detailed structure of the active pockets and/or the reactivity of the central cysteine residues may be the basis of the selectivity.

3.4. Menadione inhibits both PTEN and pAkt phosphatase in intact cells

In the experiment shown in Fig. 6, PTEN-deficient PC3 cells were artificially transfected with myc-tagged Akt (myc-Akt). As expected, the transfected myc-Akt was highly phosphorylated, which was reduced gradually when PI 3-kinase activity in the cells was blocked by LY294002. The rate of dephosphorylation was partly inhibited by menadione, indicating that the PTEN-independent degradation of PtdIns-3,4,5-P_3 and/or the activities of Ser/Thr phosphatases that target Akt were inhibited directly or indirectly. Transfection of flag-tagged PTEN (flag-PTEN) together with myc-Akt decreased

pSer-473 and pThr-308, probably by catalyzing the breakdown of PtdIns-3,4,5-P_3. The effect of flag-PTEN was inhibited by menadione, suggesting that menadione possesses the ability to inhibit PTEN in intact cells.

4. Discussion

In the present study, we showed that menadione activates Akt in CHO cells. The effect may not be due to activation of PI 3-kinase because menadione did not produce the active complex of PI 3-kinase in the cells (Fig. 3). As an alternative mechanism, we showed that menadione inhibits the lipid phosphatase PTEN in both cell-free and intact-cell systems (Fig. 4c and 6). As the inhibition of PTEN is expected to induce accumulation of PtdIns-3,4,5-P_3, it is not surprising that an inhibitor of the lipid phosphatase activates Akt. In contrast to our results in CHO cells, it is reported in several cell lines that menadione activates PI 3-kinase through the activation of EGFR. In such cells, the inhibition of PTEN and the activation of PI 3-kinase are considered to act synergisticallly in the menadione-induced activation of Akt.

In CHO cells, an inhibitor of EGFR did not attenuate the menadione-induced activation of Akt (Fig. 1B). Inhibition of PTEN is, however, not the sole mechanism of the EGFR-independent activation, because menadione decreased the rate of Akt inactivation even in cells lacking PTEN (Fig. 6). Although the regulatory mechanism of Ser/Thr phosphatase that inactivates Akt is not fully understood, the results of the present study suggest that some tyrosine phosphatase is included in the regulation. The potent action of menadione on Akt may be based on both the accelerated activation

through inhibition of PTEN and decelerated inactivation probably through inhibition of some tyrosine phosphatases.

PTEN has been shown to be reversibly inactivated by hydrogen peroxide in cell-free and intact-cell systems [28-30]. This effect of hydrogen peroxide accompanied the mobility shift of PTEN [28]. As quinones including menadione are expected to produce reactive oxygen species in intact cells, menadione may inhibit PTEN in a similar mechanism to hydrogen peroxide. However, we observed that 30 μM menadione activated Akt without causing the mobility shift of PTEN in CHO cells (data not shown). Furthermore, a potent redox cycler 1,4-benzoquinone did not activate Akt in the cells (data not shown). Thus production of reactive oxygen species may not be the mechanism leading to the PTEN inactivation in the cells. Menadione has been suggested to inhibit Cdc25A by binding to the critical cysteine residue within the signature motif [17,19,20]. Menadione may inhibit PTEN in a similar mechanism.

Naphthoquinone derivatives, including menadione, have been examined extensively for their inhibitory effects on the proliferation of tumor cells both in vivo and in vitro. The mechanism of growth arrest has been attributed to inhibition of Cdc25, prolonged phosphorylation of Erk, and increased oxidative stress. One goal of these studies is the clinical use of naphthoquinone derivatives in cancer treatment. We reported that a naphthoquinone derivative, shikonin, inhibits both PTEN and protein tyrosine phosphatases [24]. In the present study, a simple 1,4-naphthoquinone derivative, menadione, was found to inhibit PTEN in both cell-free and intact-cell systems. PTEN is a tumor suppressor, mutation of which is observed in about 50% of glioblastoma,

endometrial carcinoma, prostate carcinoma, and melanoma cases [25]. Germline mutations in PTEN are observed in Cowden disease and Bannayan-Zonana syndrome, which are characterized by increased risk of malignant breast and thyroid tumors. Thus, naphthoquinones are anticipated to have unfavorable effects in PTEN-positive cells. Further studies are required to determine whether the compounds have different effects on PTEN-positive and negative carcinoma cells.

References

[1]J.W. Suttie, Synthesis of vitamin K-dependent proteins, FASEB J. 7 (1993) 445-452.

[2]B. Furie, B.A. Bouchard, B.C. Furie, Vitamin K-dependent biosynthesis of gamma-carboxyglutamic acid, Blood 93 (1999) 1798-1808.

[3]K.N. Prasad, J. Edwards-Prasad, A. Sakamoto, Vitamin K_3 (menadione) inhibits the growth of mammalian tumor cells in culture, Life Sci. 29 (1981) 1387-1392.

[4]V. Noto, H.S. Taper, J. Yi-Hua, J. Janssens, J. Bonte, W.D. Loecker, Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K_3) treatment on human tumor cell growth in vitro. I. Synergism of combined vitamin C and K_3 action, Cancer 63 (1989) 901-906.

[5]E.O. Ngo, T.P. Sun, J.Y. Chang, C.C. Wang, K.H. Chi, A.L. Cheng, L.M. Nutter, Menadione-induced DNA damage in a human tumor cell line, Biochem. Pharmacol. 42 (1991) 1961-1968.

[6]F.Y. Wu, N.T. Chang, W.J. Chen, C.C. Juan, Vitamin K_3-induced cell cycle arrest and apoptotic cell death are accompanied by altered expression of c-fos and c-myc in nasopharyngeal carcinoma cells, Oncogene 8 (1993) 2237-2244.

[7]H. Wefers, H. Sies, Hepatic low-level chemiluminescence during redox cycling of menadione and the menadione-glutathione conjugate: Relation to glutathione and NAD(P)H:quinone reductase (DT-diaphorase) activity, Arch. Biochem. Biophys. 244 (1983) 568-578.

[8]Y. C. Chai, S. Hendrich, J.A. Thomas, Protein S-thiolation in hepatocytes stimulated

by *t*-butyl hydroperoxide, menadione, and neutrophils, Arch. Biochem. Biophys. 310 (1994) 264-272.

[9]K.A. Vallis, J. Reglinski, M. Garner, M.M. Bridgeman, C.R. Wolf, Menadione-resistant Chinese hamster ovary cells have an increased capacity for glutathione synthesis, Br. J. Cancer 76 (1997) 870-877.

[10]Y. Nishikawa, B.I. Carr, M. Wang, S. Kar, F. Finn, P. Dowd, Z.B. Zheng, J. Kerns, S. Naganathan, Growth inhibition of hepatoma cells induced by vitamin K and its analogs, J. Biol. Chem. 270 (1995) 28304-28310.

[11]R. Ni, Y. Nishikawa, B.I. Carr, Cell growth inhibition by a novel vitamin K is associated with induction of protein tyrosine phosphorylation, J. Biol. Chem. 273 (1998) 9906-9911.

[12]K. Abdelmohsen, P.A. Gerber, C. von Montfort, H. Sies, L.O. Klotz, Epidermal growth factor receptor is a common mediator of quinone-induced signaling leading to phosphorylation of connexin-43: role of glutathione and tyrosine phosphatases, J. Biol. Chem. 278 (2003) 38360-38367.

[13]J. Markovits, T.P. Sun, C.C. Juan, G.C. Ju, F.Y. Wu, Menadione (vitamin K_3) enhances the mitogenic signal of epidermal growth factor via extracellular signal-regulated kinases, Int. J. Oncol. 13 (1998) 1163-1170.

[14]S. Osada, S. Saji, K. Osada, Critical role of extracellular signal-regulated kinase phosphorylation on menadione (vitamin K_3) induced growth inhibition, Cancer, 91 (2001) 1156-1165.

[15]L.O. Klotz, P. Patak, N. Ale Agha, D.P. Buchczyk, K. Abdelmohsen, P.A. Gerber, C.

von Montfort, H. Sies, 2-Methyl-1,4-naphthoquinone, vitamin K(3), decreases gap-junctional intercellular communication via activation of the epidermal growth factor receptor/extracellular signal-regulated kinase cascade, Cancer Res. 62 (2002) 4922-4928.

[16]J.I. Beier, C. von Montfort, H. Sies, L.O. Klotz, Activation of ErbB2 by 2-methyl-1,4-naphthoquinone (menadione) in human keratinocytes: Role of EGFR and protein tyrosine phosphatases, FEBS Lett. 580 (2006) 1859-1864.

[17]S.W. Ham, H.J. Park, D.H. Lim, Studies on menadione as an inhibitor of the cdc25 phosphatase, Bioorg. Chem. 25 (1997) 33-36.

[18]K. Tamura, E.C. Southwick, J. Kerns, K. Rosi, B.I. Carr, C. Wilcox, J.S. Lazo, Cdc25 inhibition and cell cycle arrest by a synthetic thioalkyl vitamin K analogue, Cancer Res. 60 (2000) 1317-1325.

[19]B.I. Carr, Z. Wang, S. Kar, K vitamins, PTP antagonism, and cell growth arrest, J. Cell. Physiol. 193 (2002) 263-274.

[20]J. Kerns, S. Naganathan, P. Dowd, F.M. Finn, B. Carr, Thioalkyl Derivatives of vitamin K_3 and vitamin K_3 oxide inhibit growth of Hep3B and HepG2 cells, Bioorg. Chem. 23 (1995) 101-108.

[21]T. Maehama, J.E. Dixon, The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate, J. Biol. Chem. 273 (1998) 13375-13378.

[22]E.B. Fauman, M.A. Saper, Structure and function of the protein tyrosine phosphatases, Trends Biochem. Sci. 21 (1996) 413-417.

[23]M.P. Wymann, L. Pirola, Structure and function of phosphoinositide 3-kinases, Biochim. Biophys. Acta 1436 (1998) 127-150.

[24]K. Nigorikawa, K. Yoshikawa, T. Sasaki, E. Iida, M. Tsukamoto, H. Murakami, T. Maehama, K. Hazeki, O. Hazeki, A naphthoquinone derivative, shikonin, has insulin-like actions by inhibiting both phosphatase and tensin homolog deleted on chromosome 10 and tyrosine phosphatases, Mol. Pharmacol. 70 (2006) 1143-1149.

[25]T. Maehama, J.E. Dixon, PTEN: a tumour suppressor that functions as a phospholipid phosphatase, Trends Cell Biol. 9 (1999) 125-128.

[26]F.Y. Wu, T.P. Sun, Vitamin K_3 induces cell cycle arrest and cell death by inhibiting Cdc25 phosphatase, Eur. J. Cancer 35 (1999) 1388-1393.

[27]J.O. Lee, H. Yang, M.M. Georgescu, A. Di Cristofano, T. Maehama, Y. Shi, J.E. Dixon, P. Pandolfi, N.P. Pavletich, Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association, Cell 99 (1999) 323-334.

[28]S.R. Lee, K.S. Yang, J. Kwon, C. Lee, W. Jeong, S.G. Rhee, Reversible inactivation of the tumor suppressor PTEN by H_2O_2, J. Biol. Chem. 277 (2002) 20336-20342.

[29]N.R. Leslie, D. Bennett, Y.E. Lindsay, H. Stewart, A. Gray, C.P. Downes, Redox regulation of PI 3-kinase signalling via inactivation of PTEN, EMBO J. 22 (2003) 5501-5510.

[30]J. Kwon, S.R. Lee, K.S. Yang, Y. Ahn, Y.J. Kim, E.R. Stadtman, S.G. Rhee, Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors, Proc. Natl. Acad. Sci. USA, 101 (2004) 16419-16424.

Figure Legends

Fig. 1. EGFR-independent activation of Akt by menadione. A, CHO-IR cells were incubated with 300 nM wortmannin (Wort) or 30 μM LY294002 (LY) at 37°C for 5 min. The cells were further incubated for 4 min with addition of 30 μM menadione (M) or 100 nM insulin (I). The cell lysates were subjected to Western blotting analysis with the indicated antibodies. B, CHO-IR cells were incubated in the presence or absence of 300 nM AG1478 for 30 min. The cells were further incubated with 50 ng/ml EGF for 5 min (E), with 100 nM insulin for 5 min (I), or with 30 μM menadione for 15 min. The cell lysates were subjected to Western blotting analysis with the indicated antibodies. C, PC12 cells were incubated for 30 min with the indicated concentrations of AG1478. The cells were further incubated for 5 min with or without addition of EGF. The cell lysates were subjected to Western blotting analysis with anti-pErk, anti-pAkt, or anti-Akt.

Fig. 2. Inability of menadione to prevent IRβ dephosphorylation. In A, CHO-IR cells were first incubated for 4 min with 100 nM insulin. The cells were incubated further for 0, 1, 5, 10, or 20 min, where indicated as “without washout.” Alternatively, the cells were washed quickly with acidified buffer (pH 6.0) to remove insulin, and then incubated in fresh buffer (pH 7.4) for 1, 5, 10, or 20 min, where indicated as “after washout.” The cell lysates were subjected to Western blotting analysis with anti-pTyr or anti-IRβ. In B, the cells were incubated with 30 μM menadione for 15 min before being treated as in A. When the cells were washed with acidified buffer, 30 μM menadione

was included in the fresh buffer. Similar results were obtained in a repeated experiment.

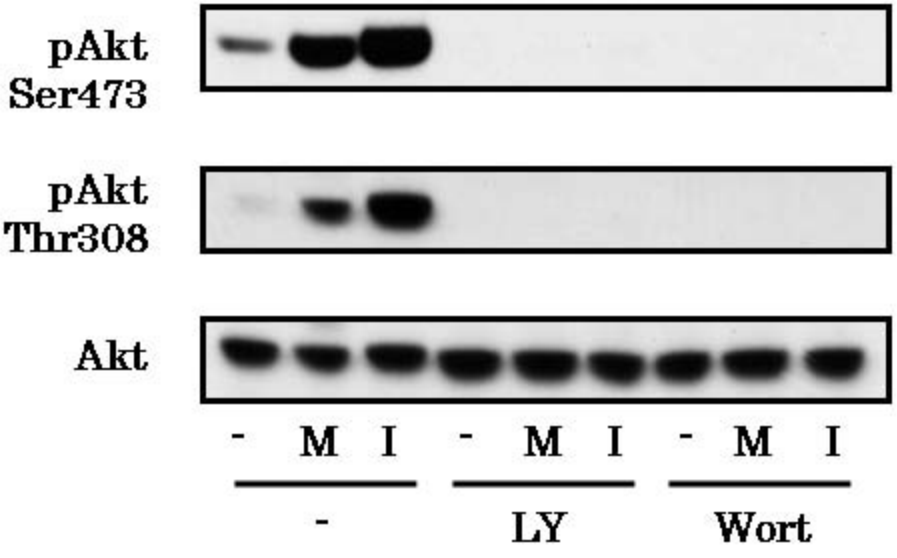
Fig. 3. Inability of menadione to activate PI 3-kinase. CHO-IR cells were incubated with or without 30 μM menadione for 15 min, and then for 5 min with addition of the indicated concentrations of insulin. The cell lysates were subjected to the immunoprecipitation with anti-pTyr antibody. The immune complex was analyzed for PI 3-kinase activity with phosphatidylinositol as the substrate. Lipids were extracted and separated on thin-layer chromatography plates. The radioactivity in the PtdIns-3-P fraction was visualized by autoradiography.

Fig. 4. Inhibition of PTEN by menadione. In A, CHO-IR cells were first incubated for 4 min with 100 nM insulin. The cells were incubated for a further 0, 1, 2, 3, or 5 min, where indicated as "without Wort." Alternatively, 300 nM wortmannin was added to the cells and incubation was continued for a further 1, 2, 3, or 5 min, where indicated as "with Wort." The cell lysates were subjected to Western blotting analysis with the indicated antibodies. In B, the cells were incubated with 30 μM menadione for 15 min before being treated as in A. The paired result shown in A and B is a representative of three separate experiments. C, Recombinant PTEN was incubated at 37°C for 5 min with various concentrations of menadione. The mixture was incubated for a further 5 min with addition of diC_{16}-PtdIns-3,4,5-P_3. The amount of released phosphate was determined with GREEN Reagent. The result from three separate experiments is shown as mean ± SEM.

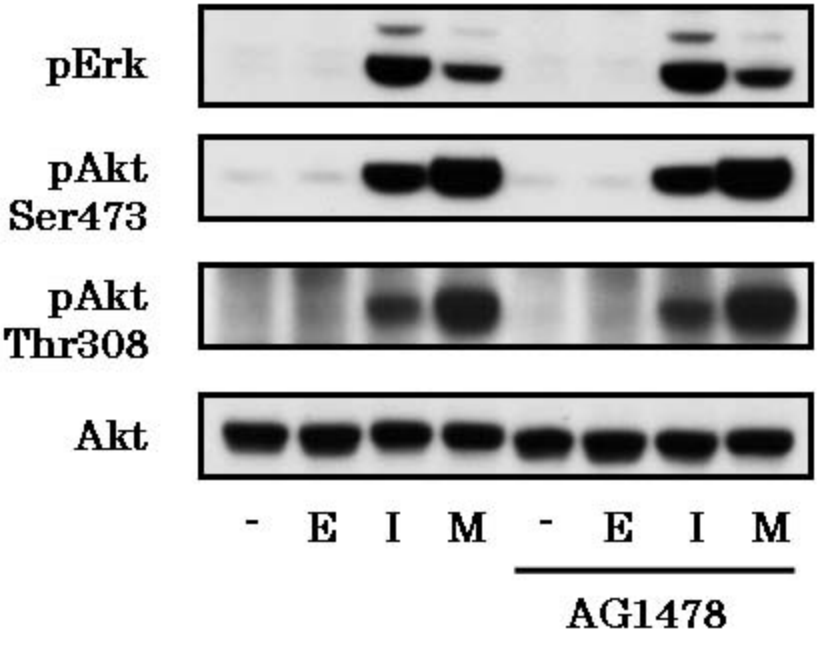
Fig. 5. Effects of menadione on pNPP phosphatase activities. Cdc25A, VHR, PTP1B, SHP1, or SHP2 was incubated at 30°C for 10 min with various concentrations of menadione. After addition of 5 mM pNPP, phosphatase reaction was allowed to proceed for 10 min. The amount of p-nitrophenol produced was determined from the absorbance at 405 nm. For each enzyme, the result from three separate experiments is shown as mean ± SEM. The activity in the absence of menadione was 0.31 ± 0.103 μmol/μg/10 min (Cdc25A), 63 ± 8.8 μmol/μg/10 min (VHR), 1.9 ± 0.07 mmol/μg/10 min (PTP1B), 0.20 ± 0.046 mmol/μg/10 min (SHP1), or 5.0 ± 0.33 μmol/μg/10 min (SHP2). The hydrolysis by PTEN was measured by incubating the mixture at 20°C for 15 hour. PTEN hydrolyzed 8.3 μmol/μg of pNPP during the period. The vertical bars of the result of PTEN indicate the difference of duplicate determinations.

Fig. 6. Effects of menadione on Akt dephosphorylation in PC3 cells. PC3 cells were transfected with myc-tagged Akt together with flag-tagged PTEN or empty vector. The cells were incubated in the presence or absence of 30 μM menadione for 15 min, and then with or without addition of 50 μM LY294002 for the indicated periods. A, The cell lysates were mixed with anti-myc, and the immune complexes formed were subjected to Western blotting analysis with anti-pAkt (Ser 473), anti-pAkt (Thr 308), or anti-myc. B, The cell lysates were subjected directly to Western blotting analysis with anti-PTEN or anti-myc. Similar results were obtained in a repeated experiment.

A



B



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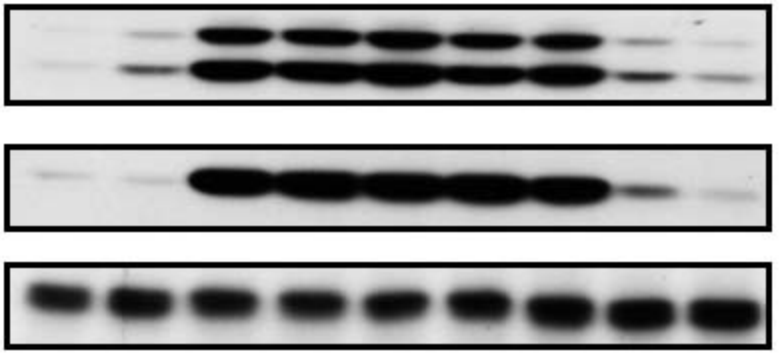


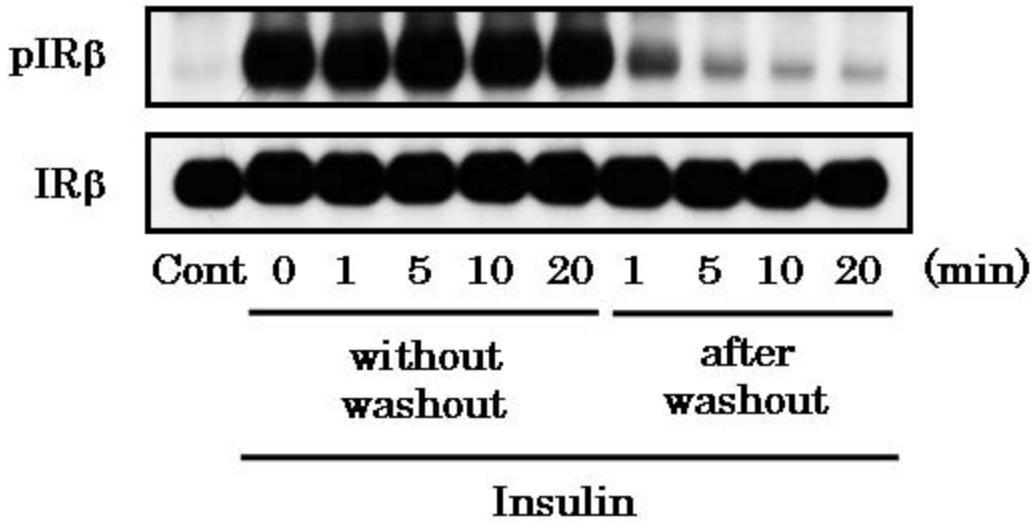
Cont	0	1	5	10	50	50	50	50

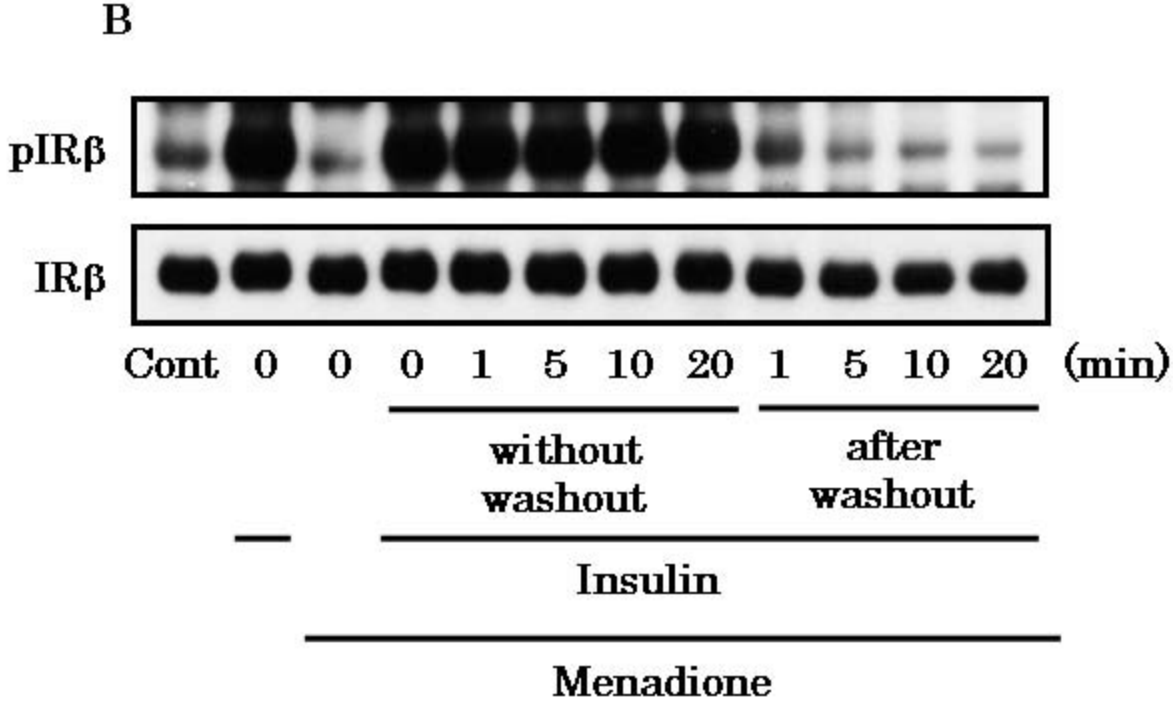
EGF (ng/ml)

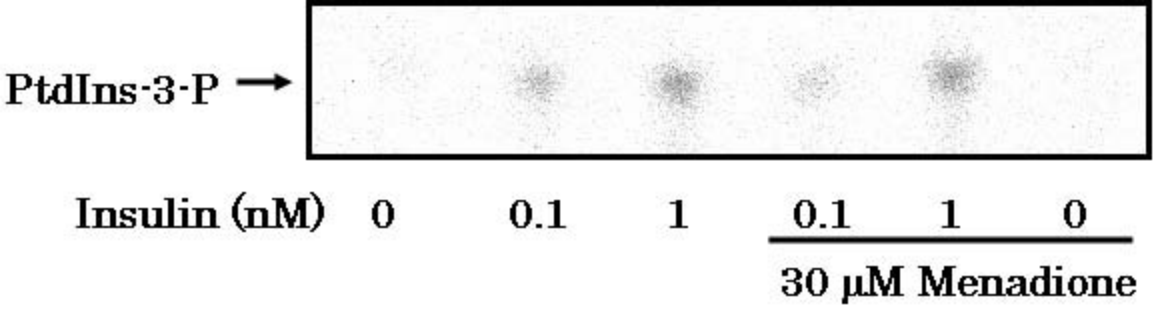
1	0	0	0	0	0.03	0.3	1

AG1478 (μM)

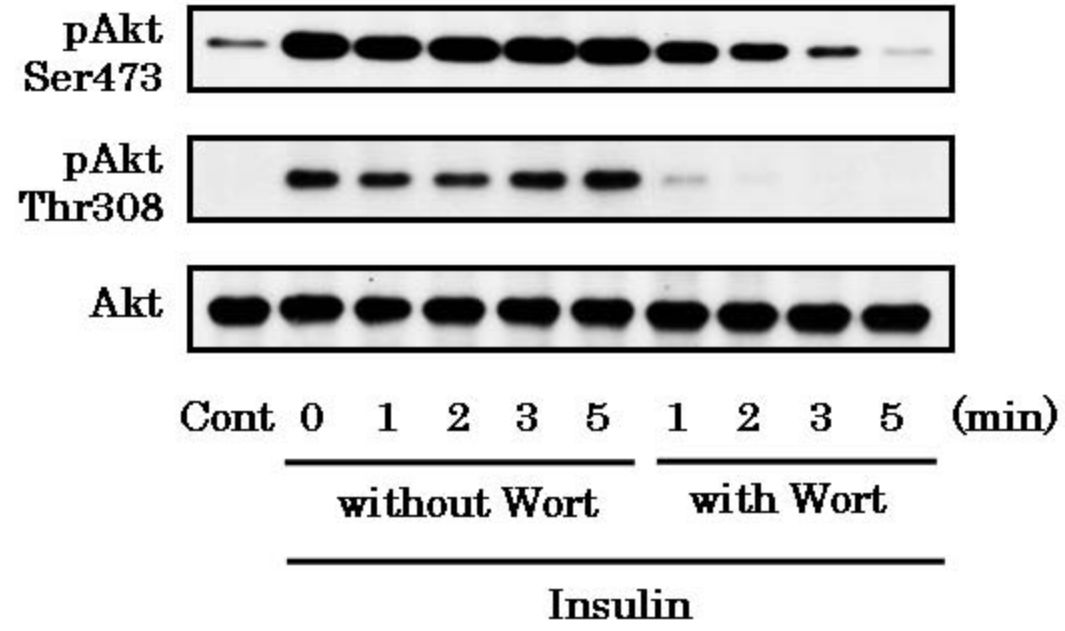
A

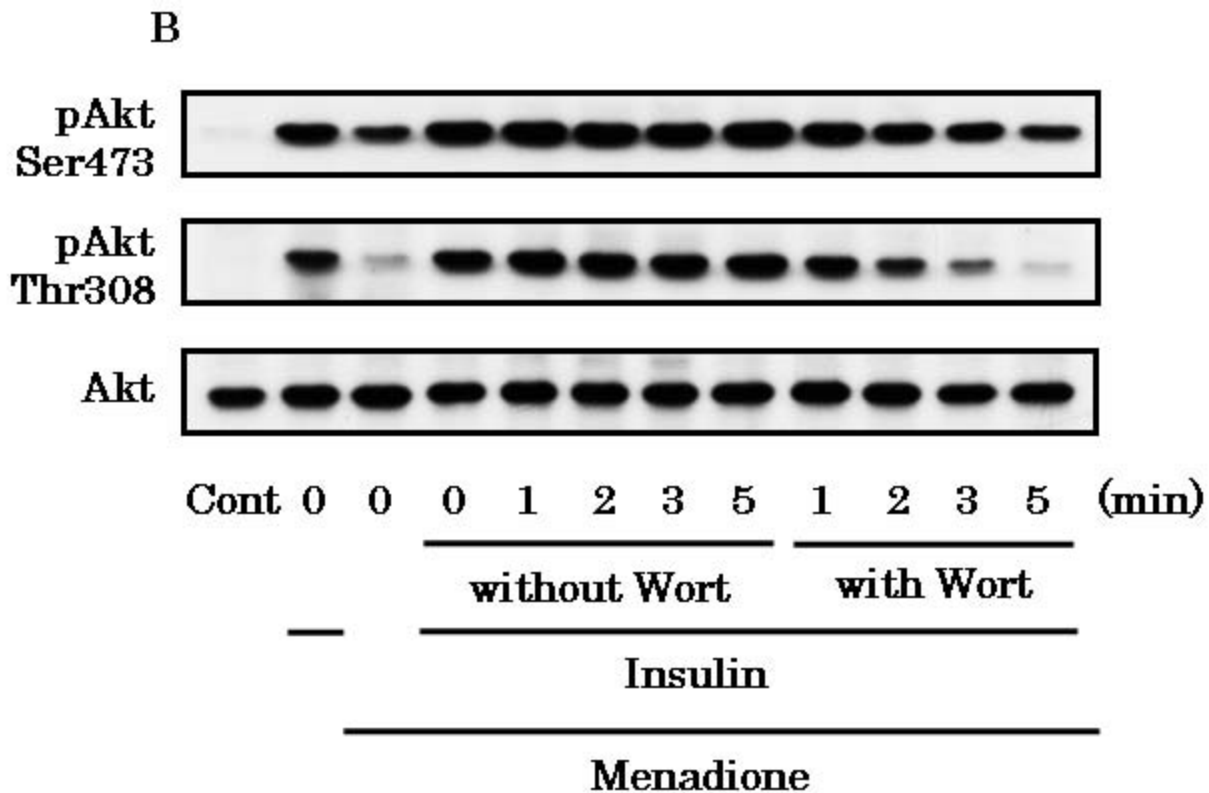

pIRβ
IRβ
Cont 0 1 5 10 20 1 5 10 20 (min)
without washout
after washout
Insulin


B
pIRβ
IRβ
Cont 0 0 0 1 5 10 20 1 5 10 20 (min)
without washout
after washout
Insulin
Menadione

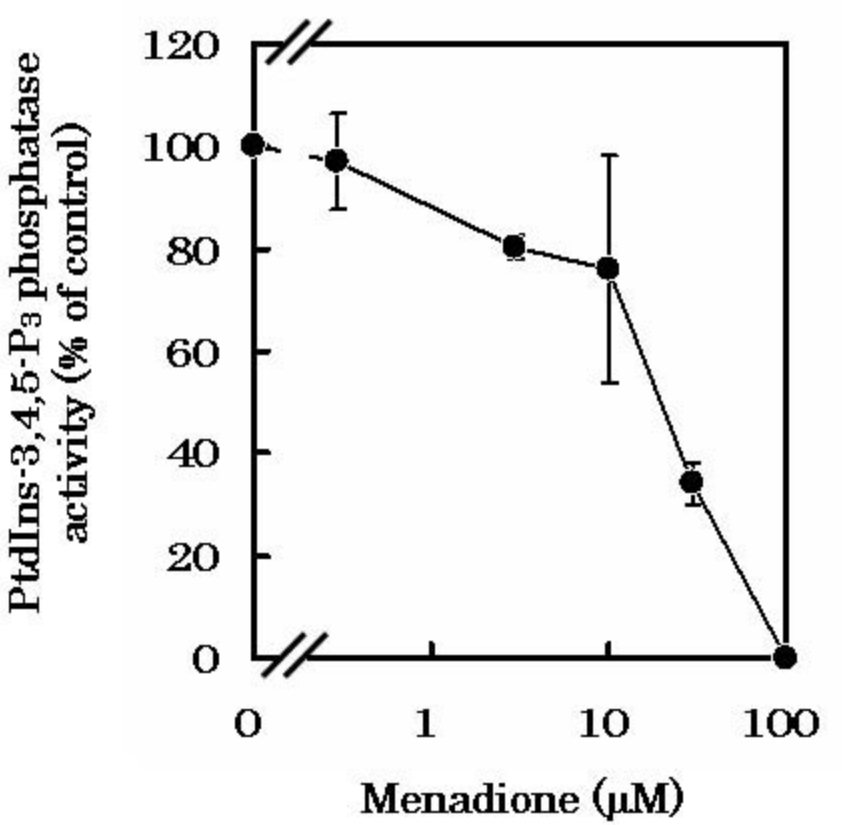

PtdIns-3-P
Insulin (nM)
0
0.1
1
0.1
1
0
30 μM Menadione

A

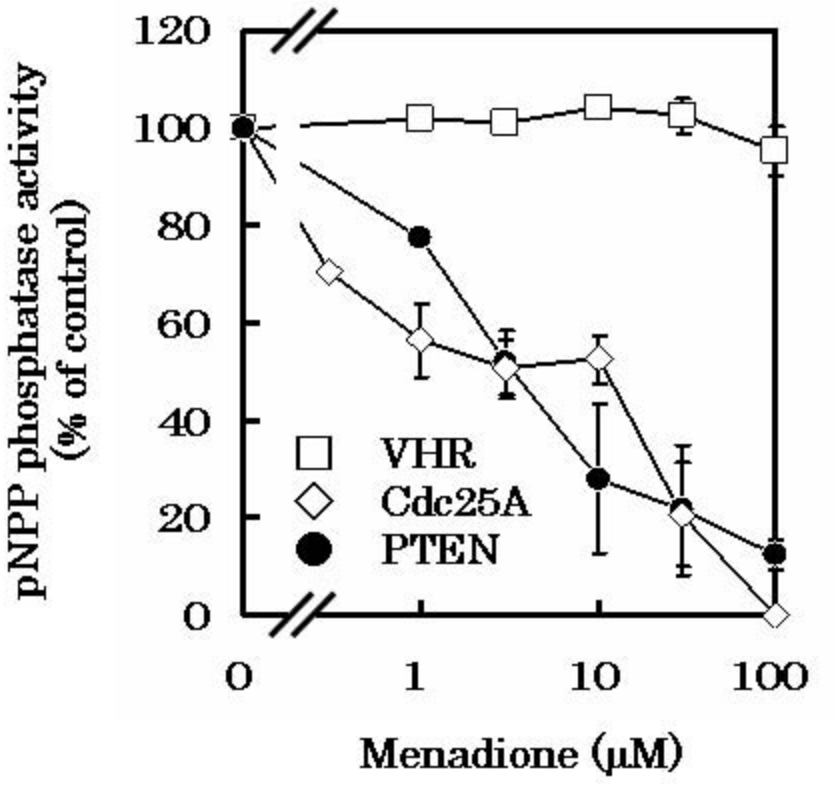

pAkt
Ser473
pAkt
Thr308
Akt
Cont 0 1 2 3 5 1 2 3 5 (min)
without Wort
with Wort
Insulin


B
pAkt
Ser473
pAkt
Thr308
Akt
Cont 0 0 0 1 2 3 5 1 2 3 5 (min)
without Wort
with Wort
—
Insulin
Menadione

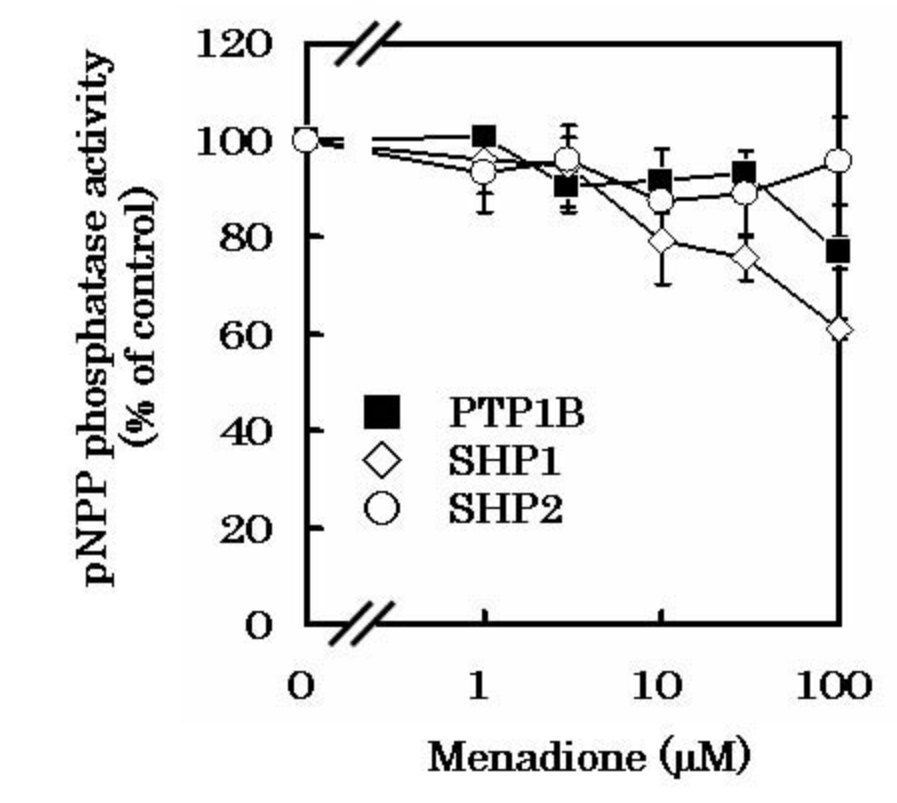
C

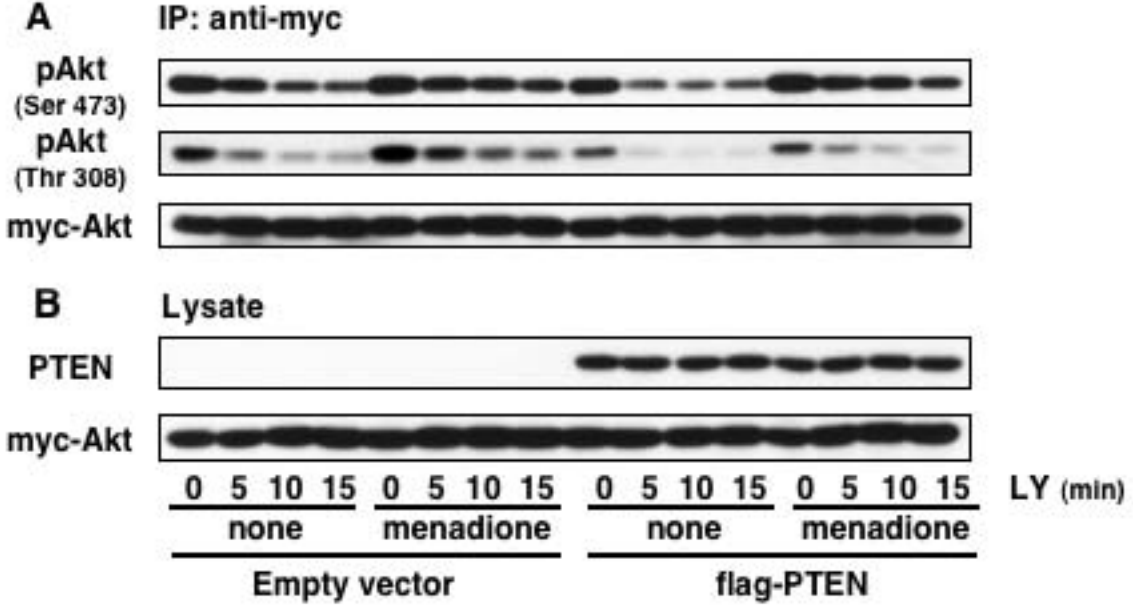

PtdIns-3,4,5-P3 phosphatase activity (% of control)
120
100
80
60
40
20
0
0
1
10
100
Menadione (μM)

A


120
100
80
60
40
20
0
pNPP phosphatase activity
(% of control)
VHR
Cdc25A
PTEN
0
1
10
100
Menadione (μM)

B


120
100
80
60
40
20
0
pNPP phosphatase activity
(% of control)
PTP1B
SHP1
SHP2
0
1
10
100
Menadione (μM)


A
IP: anti-myc
pAkt
(Ser 473)
pAkt
(Thr 308)
myc-Akt
B
Lysate
PTEN
myc-Akt
0 5 10 15 0 5 10 15 0 5 10 15 0 5 10 15 LY (min)
none
menadione
none
menadione
Empty vector
flag-PTEN