0026-895X/06/7003-1143–1149$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 70:1143–1149, 2006

Vol. 70, No. 3
25809/3137898
Printed in U.S.A.

A Naphthoquinone Derivative, Shikonin, Has Insulin-Like Actions by Inhibiting Both Phosphatase and Tensin Homolog Deleted on Chromosome 10 and Tyrosine Phosphatases

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Received April 16, 2006; accepted June 27, 2006

ABSTRACT

The 1,4-naphthoquinone derivative, shikonin, has been shown to increase glucose uptake by adipocytes and myocytes with minor effects on protein tyrosine phosphorylation in the cells (*Biochem Biophys Res Commun* **292:**642–651, 2002). The present study was performed to examine the mechanism of this action of shikonin. Shikonin inhibited the phosphatidylinositol 3,4,5-triphosphate (PtdIns-3,4,5-P_3) phosphatase activity of recombinant phosphatase and tensin homolog deleted on chromosome 10 (PTEN) with an IC_{50} value of 2.7 μM. Shikonin induced marked accumulation of PtdIns-3,4,5-P_3 and activation of protein kinase B (PKB) in Chinese hamster ovary cells expressing insulin receptors. In addition to its effect on PTEN, shikonin was found to inhibit several protein phosphatases in cell-free systems. Its effect on tyrosine phosphorylation in intact cells was far weaker than that of pervanadate, a widely used tyrosine phosphatase inhibitor, despite the observation that the effect of shikonin on PKB was more potent than that of pervanadate. These results suggested that the inhibition of PTEN provides a clue to its potent insulin-like actions. We also found that naphthoquinones, including 1,2-naphthoquinone, inhibit PTEN in the cell-free system, which suggested that the effect on PTEN (and thus the effect on phosphatidylinositol 3-kinase signaling) should be taken into account when examining the pharmacological actions of naphthoquinone derivatives.

Binding of insulin to its specific receptors causes tyrosine phosphorylation and activation of the receptors, which in turn induces the activation of downstream signaling molecules. Phosphoinositide 3-kinase (PI 3-kinase) is one of the factors involved in transduction of the insulin signal from the receptors (Wymann and Pirola, 1998). The SH2 domains of the regulatory subunit of PI 3-kinase bind to the phosphorylated tyrosine residues in the consensus sequence, YXXM. This binding activates PI 3-kinase and results in the accumulation of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2. The machinery downstream of PI 3-kinase includes a serine/threonine kinase protein kinase B (PKB, also known as Akt), the activity of which is up-regulated by the products of PI 3-kinase (Alessi and Downes, 1998). Overexpression of a constitutively active form of PKB has been reported to lead to increased glucose uptake and GLUT4 translocation in 3T3L1 adipocytes (Kohn et al., 1996).

The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is an enzyme that dephosphorylates the D3 position of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 (Maehama and Dixon, 1998; Cantley and Neel, 1999). Overexpression of the lipid phosphatase reduces the insulin-induced accumulation of PtdIns-3,4,5-P_3 in 293 cells (Maehama and Dixon, 1998). Infection with adenovirus expressing PTEN in 3T3L1 adipocytes attenuates both the insulin-induced glucose uptake and the Akt activation (Nakashima et al., 2000). Microinjection of an anti-PTEN antibody potentiates the insulin-induced events (Ono et al., 2001). Thus, PTEN is regarded as a negative regulator of insulin signaling.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.025809.

ABBREVIATIONS: PI 3-kinase, phosphoinositide 3-kinase; CHO-IR cells, Chinese hamster ovary cells expressing insulin receptors; IRβ, the β subunit of insulin receptor; pNPP, *p*-nitrophenyl phosphate; PKB, protein kinase B; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; WGA, wheat germ agglutinin; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride.

Shikon is the dried root of the plant *Lithospermum erythrorhizon* (Siebold et Zucc.), the extract of which is used in traditional Chinese medicine for the treatment of macular eruptions, measles, sore throat, carbuncles, and burns (Papageorgiou et al., 1999; Chen et al., 2002). With regard to the molecular basis of its actions, shikonin, a naphthoquinone pigment isolated from the root (or its derivatives), have been reported to possess a variety of pharmacological activities, including anti-inflammatory and antitumor activities (Sankawa et al., 1977; Tanaka et al., 1986; Hisa et al., 1998; Hashimoto et al., 2002). Kamei et al. (2002) found another action of shikonin while screening for small molecules with insulin-like actions. The study showed that shikonin effectively increases the glucose uptake of 3T3L1 adipocytes and rat cardiomyocytes. This effect of shikonin is not accompanied by activation of insulin receptor-tyrosine kinase.

Several studies have been performed to clarify the site of action of shikonin in the signal transduction system of cells. In neutrophils, a shikonin derivative, acetylshikonin, has been reported to reduce *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced protein tyrosine phosphorylation by 90% (Wang et al., 1997). This compound inhibits PtdIns-4,5-P_2 breakdown and Ins-1,4,5-P_3 formation in the cells by inhibiting phospholipase C (Wang and Kuo, 1997). Shikonin has also been reported to inhibit the production of tumor necrosis factor-α by inhibiting the expression of its mRNA (Staniforth et al., 2004). Although these mechanisms may represent the basis of some actions of shikonin, they do not explain its insulin-like action. In the present study, we examined the effects of shikonin on insulin signaling using Chinese hamster ovary cells expressing insulin receptors (CHO-IR cells) as a model system. The results suggested that the inhibitory effect on PTEN provides a clue to its potent insulin-like actions.

Materials and Methods

Reagents. Shikonin was purchased from Calbiochem (San Diego, CA). Although the purity of the shikonin preparation was 98%, a highly purified preparation obtained from Wako Pure Chemicals (Tokyo, Japan) showed similar results to the present study. 1,2-Naphthoquinone and 5,8-dihydroxy-1,4-naphthoquinone were from Tokyo Kasei Kogyo (Tokyo, Japan). Insulin, sodium orthovanadate, bovine serum albumin (fatty acid-free), Ni-CAM HC Resin, and anti-myc antibody (9E10) were from Sigma (St. Louis, MO). Wortmannin and LY294002 were from Kyowa Medex (Tokyo, Japan) and Cayman Chemical (Ann Arbor, MI), respectively. $^{32}P_i$ and [γ-^{32}P]ATP were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Monoclonal antibodies against pThr308 and pSer473 of PKB and polyclonal antibodies against PTEN and phospho-PTEN (Ser308) were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-PKB antibody, polyclonal anti-IRβ antibody, and monoclonal anti-phosphotyrosine antibody (PY99) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG agarose was from American Qualex Antibodies (San Clemente, CA). pGEX-6P-1, PreScission protease, and PD-10 column were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). pQE-30 expression vector was from QIAGEN (Hilden, Germany). Pervanadate was prepared by mixing sodium orthovanadate and hydrogen peroxide at equimolar concentrations. Naphthoquinone compounds were dissolved in dimethyl sulfoxide before use, and all experiments were performed in the presence of <0.5% dimethyl sulfoxide.

Cell Lines. CHO-IR cells were kind gifts from Dr. Yosuke Ebina (Ehime University, Matsuyama, Japan) and were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

Recombinant Proteins. cDNAs encoding PTP1B, SHP-1, Cdc25A (GenBank accession numbers NM_002827, NM_002831, and NM_001789, 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 or pQE-30. Glutathione transferase-fused or 6×His-tagged proteins were expressed in *Escherichia coli* and adsorbed onto glutathione-Sepharose 4B or high-capacity nickel chelate affinity matrix 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 M dithiothreitol. Histidine-tagged proteins were eluted from the nickel-CAM beads with 250 mM imidazole. The eluate was changed to Tris-buffered saline containing 1 M dithiothreitol using a PD-10 column before storage at −80°C.

Immunoprecipitation and Immunoblotting. The CHO-IR cells were starved of serum and 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. After incubation with the indicated compounds at 37°C, the buffer was removed, and the reactions were stopped by adding 1 ml of ice-cold phosphate-buffered saline containing 10 mM NaF and 1 mM sodium orthovanadate. The cells were then 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, 20 μM (4-amidinophenyl)-methansulfonyl fluoride, 2 μM leupeptin, 2 μM pepstain, and 1% Nonidet P-40. After centrifugation (15,000 rpm for 10 min), aliquots of the supernatant were mixed with SDS-polyacrylamide gel electrophoresis sample buffer and boiled for 5 min. The peptides were separated by SDS-polyacrylamide gel electrophoresis 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 (PerkinElmer Life and Analytical Sciences). For immunoprecipitation, the cell lysates were incubated with anti-phosphotyrosine or anti-myc antibody for 1.5 h at 4°C and then mixed with anti-mouse IgG agarose. The immune complex was washed three times with lysis buffer and boiled for 5 min in sample buffer. The peptides were separated and analyzed by immunoblotting as described above.

Protein Kinase B Activity. The CHO-IR cells were cultured on six-well plates and transfected with myc-tagged PKB in the expression vector, pCMV5, as described previously (Kubo et al., 2005). The transfected cells were starved of serum before treatment with insulin or shikonin. The cells were solubilized in 400 μl of lysis buffer and then mixed with anti-myc antibody. The immune complex was washed twice and incubated at 30°C for 15 min in 20 μl of 50 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 30 μM Crosstide, and 100 μM [γ-^{32}P]ATP (0.25 μCi/ml). Aliquots of the incubation mixture were spotted onto P81 anion exchange paper (Whatman, Brentford, UK) to absorb the peptide substrate. The paper was washed vigorously with 1% phosphoric acid, and then the associated radioactivity was determined.

Accumulation of PtdIns-3,4,5-P_3. CHO-IR cells, cultured on six-well plates, were starved of serum and labeled with $^{32}P_i$ (50 μCi/ml) for 1 h. The medium was replaced with incubation buffer, and the cells were stimulated with insulin or shikonin at 37°C. The buffer was removed before the cells were scraped off in 0.6 ml of 8% $HClO_4$. The suspension was mixed with 2.25 ml of chloroform/methanol [1:2 (v/v)] and the extracted lipids were separated onto oxalate-impregnated thin-layer chromatography plates as described previously (Takasuga et al., 1999).

PI 3-Kinase Activity. CHO-IR cells, cultured on six-well plates, were starved of serum and treated with insulin or shikonin. The cells were then solubilized in buffer consisting of 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 20 μM (4-amidinophenyl)-methansulfonyl fluoride, 2 μM leupeptin, 2 μM pepstain, and 1% Nonidet P-40. After centrifugation at 15,000 for 20 min, the supernatant was subjected to immunoprecipitation with anti-pTyr antibody. PI 3-kinase activity in the immune complex was determined using phosphatidylinositol as the substrate.

Lipid Phosphatase Activity. PTEN (50 ng) was preincubated at 37°C for 5 min in 10 μl of buffer consisting of 0.1 M Tris-HCl, pH 8.0, 10 mM dithiothreitol, 0.25% octyl glucoside, and 0.1 μg/μl bovine serum albumin, in the presence or absence of the indicated compounds. The phosphatase reaction was started by addition of 0.1 mM diC16-PtdIns-3,4,5-P_3 and was stopped after 5 min by the addition of 80 μl of Malachite green reagent, as described previously (Maehama et al., 2000; Schmid et al., 2004). After the color was developed for 15 min, absorbance at 620 nm was measured. The amount of phosphate released was quantified using standard solutions of inorganic phosphate.

pIRβ Phosphatase Activity. Phosphorylated IRβ was semipurified from insulin-treated CHO-IR cells using wheat germ agglutinin (WGA)-agarose (Vector Laboratories, Burlingame, CA). His-PTP1B (200 ng) was incubated at 37°C for 10 min in the presence or absence of shikonin in a buffer consisting of 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 1 mM dithiothreitol. The tyrosine phosphatase reaction was started by the addition of pIRβ-bound WGA-agarose beads. After 10 min, the reaction was stopped by the addition of chilled buffer consisting of 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.1 mM sodium orthovanadate, and 1% Nonidet P-40. The beads were washed with the same buffer and boiled in SDS sample buffer for 5 min. The tyrosine phosphorylation of IRβ was analyzed by immunoblotting.

p-Nitrophenyl Phosphate Phosphatase Activity. His-PTP1B (20 ng), His-SHP1 (500 ng), or Cdc25A (15 μg) was incubated at 37°C for 10 min in 135 μl of reaction buffer containing various concentrations of the inhibitors. The reaction buffer for the activities of PTP1B and SHP1 consisted of 100 mM sodium acetate, pH 5.5, 100 mM NaCl, and 1 mM dithiothreitol, whereas the activity of Cdc25A was assayed in buffer consisting of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM dithiothreitol. The phosphatase reaction was started by the addition of 15 μl of *p*-nitrophenyl phosphate (pNPP) solution and stopped after 10 min by the addition of 150 μl of 1 N NaOH. The concentrations of pNPP approximated the K_m values of each phosphatase: PTP1B, 1 mM; SHP1, 26 mM; and Cdc25A, 45 mM. The amount of *p*-nitrophenol produced was determined from the absorbance at 405 nm. The IC_{50} value was determined by interpolation of the percentage inhibition versus inhibitor concentration plot.

For examination of the time dependence of the action of shikonin, His-PTP1B (100 ng) was first incubated for various times with 20 μM shikonin in the presence or absence of 5 mM phenylphosphate in Tris-buffered saline containing 0.01% bovine serum albumin and 1 M dithiothreitol. The mixture was diluted 100-fold with a reaction buffer consisting of 100 mM sodium acetate, pH 5.5, 100 mM NaCl, 1 mM dithiothreitol, and 5 mM pNPP. The phosphatase reaction was allowed to proceed for 10 min and was stopped by the addition of 0.5 N NaOH. The samples incubated in the absence of phenylphosphate were diluted with reaction buffer containing 50 μM phenylphosphate. A control experiment was performed by adding shikonin at the start of the phosphatase reaction.

Results

Although shikonin has been reported to possess insulin-like actions in adipocytes and myocytes, its mechanism of action has not been clarified. Because PKB is known to play a role in insulin-induced cell events (Kohn et al., 1996; Alessi and Downes, 1998), we first examined whether shikonin has any effect on PKB in CHO-IR cells. Treatment of the cells with shikonin caused phosphorylation of PKB on both the Ser473 residue in the C-terminal hydrophobic motif and the Thr308 residue in the activation loop (Fig. 1A). The level of phosphorylation reached a maximum 10 min after the addition of shikonin, and the effect of 30 μM shikonin was as potent as 0.1 μM insulin (Fig. 1B). An inhibitor of PI 3-kinase, wortmannin, completely abolished the shikonin-induced phosphorylation and that induced by insulin. Another inhibitor, LY294002, also inhibited the effects of both insulin and shikonin (data not shown). The presence of highly expressed insulin receptors is not required for the shikonin action because the increased phosphorylation of PKB can be observed in the parent CHO cells (data not shown).

The effect of shikonin on PKB was further examined using cells transfected with myc-tagged PKB. The lysate from the cells was mixed with anti-myc antibody, and the protein kinase activity associated with the immune complex was determined. Treatment of the cells with shikonin increased the activity of myc-PKB (Fig. 1C). This effect was inhibited by the PI 3-kinase inhibitors wortmannin and LY294002 (Fig. 1D). The activity of myc-PKB correlated well with phosphorylation on the Ser473 and Thr308 residues (Fig. 1E).

PKB is phosphorylated and activated when the products of PI 3-kinase bind to the PH domain of the enzyme (Alessi and Downes, 1998). Thus, we next examined whether shikonin had any effect on PtdIns-3,4,5-P_3 accumulation in the cells. Shikonin induced marked accumulation of [^{32}P]PtdIns-3,4,5-P_3 in $^{32}P_i$-labeled cells (Fig. 2). The effects of both shikonin and insulin were abolished by wortmannin and LY294002.

The above results suggested that the action of shikonin was mediated either by increasing PtdIns-3,4,5-P_3 production or by decreasing its degradation. Thus, we first examined the effect of shikonin on PI 3-kinase activities. When the cells were treated with insulin, a significant increase in PI 3-kinase activity was observed in the immune complex with anti-pTyr antibody (Fig. 3A). Shikonin has the ability to activate PI 3-kinase, although its effect was far weaker than that of insulin. Shikonin had no effect when added directly to the assay mixture of PI 3-kinase, which was prepared from nontreated cells using an antibody against the p85 regulatory subunit of PI 3-kinase (data not shown).

Figure 3B shows the results when the total cell lysate was analyzed by Western blotting with anti-pTyr antibody. Insulin increased the intensity of a 95-kDa band, which was considered to be the β-subunit of the insulin receptor (IRβ). Shikonin caused tyrosine phosphorylation of a peptide with a mobility similar to that of IRβ. The effect of shikonin on IRβ was confirmed when the lysate was first mixed with anti-pTyr antibody, and the immune complex was analyzed with anti-IRβ antibody (Fig. 3C).

One possible explanation for the increased tyrosine-phosphorylation of IRβ was that shikonin directly influenced the autophosphorylation of IRβ. However, shikonin showed no effect on the activity of insulin receptor-tyrosine kinase, which was purified from both insulin-treated and -untreated CHO-IR cells using WGA-agarose beads (data not shown). An alternative explanation is that the effect of shikonin was mediated by inhibiting the dephosphorylation of IRβ. Therefore, we examined the effect of shikonin on PTP1B, a tyrosine

phosphatase known to dephosphorylate IRβ. As shown in Fig. 4A, shikonin inhibited the ability of recombinant PTP1B to dephosphorylate the IRβ prepared from insulin-treated CHO-IR cells. When the PTP1B activity was determined using 1 mM pNPP as the substrate, shikonin inhibited the phosphatase in a dose-dependent manner with an IC_{50} value of 17 μM (Fig. 4B). Shikonin inhibited the pNPP phosphatase activities of another tyrosine phosphatase, SHP-1, and a dual specificity phosphatase, Cdc25A (Table 1).

In the experiment shown in Fig. 4C, PTP1B was first incubated with 20 μM shikonin for various times, and the mixture was diluted by 100-fold before measurement of the pNPP phosphatase activity. Prior incubation with shikonin markedly decreased PTP1B activity in the diluted solution. The degree of inhibition was decreased when shikonin treatment was performed in the presence of a poor substrate, phenylphosphate (open symbols). Thus, the action of shikonin is considered to be irreversible and involves binding to the active center of the enzyme, as was the case in inhibition of human prostatic acid phosphatase by 4-difluoromethylphenyl phosphate (Wang et al., 1994).

The above results showed that shikonin possesses the potential ability to activate PI 3-kinase by inactivating tyrosine phosphatases in intact cells. However, it is unlikely that this action is the sole mechanism by which shikonin induces marked accumulation of PtdIns-3,4,5-P_3 in the cells. The above results showed that the effects of shikonin on activation of PI 3-kinase (Fig. 3A) and phosphorylation of IRβ (Fig. 3C) were far weaker than those of insulin. On the other hand, the effects on accumulation of PtdIns-3,4,5-P_3 (Fig. 2) and activation of PKB (Fig. 1) were comparable with, or even more effective than the effects of insulin. One possible explanation for this discrepancy is that shikonin prevents the degradation of PtdIns-3,4,5-P_3. Thus, we next examined its effect on PTEN, a PtdIns-3,4,5-P_3 phosphatase. As shown in Fig. 5A, the expression and phosphorylation status of PTEN was unaffected by the shikonin treatment. In the experiment shown in Fig. 5B, recombinant PTEN was incubated with PtdIns-3,4,5-P_3, and the liberation of phosphate was determined by Malachite green assay. Shikonin inhibited the lipid

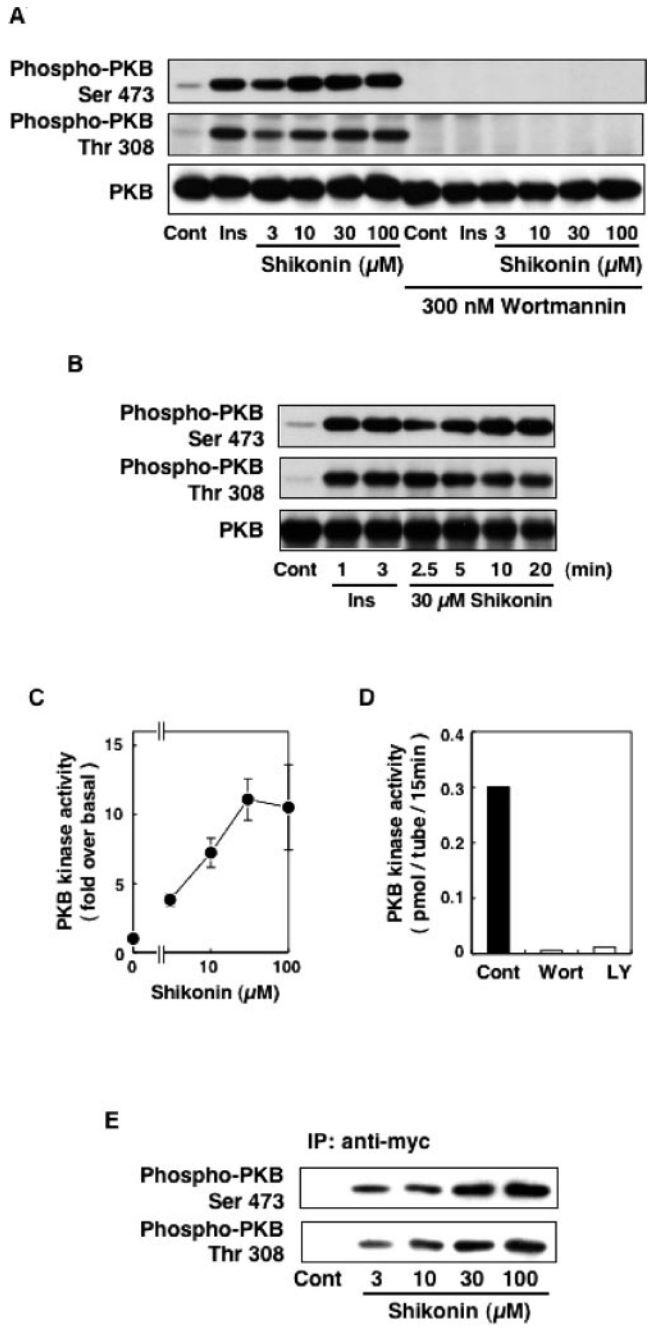

Fig. 1. Effects of shikonin on protein kinase B in CHO-IR cells. A, CHO-IR cells were incubated in the presence or absence of 300 nM wortmannin at 37°C for 10 min. The cells were further incubated for 5 min with various concentrations of shikonin or for 3 min with 100 nM insulin. The lysate from the cells was subjected to Western blotting analysis with the indicated antibodies. B, CHO-IR cells were incubated with 30 μM shikonin or 100 nM insulin for the indicated times. The cell lysate was subjected to Western blotting analysis with the indicated antibodies. C, D, and E, CHO-IR cells were transfected with myc-tagged PKB in the expression vector, pCMV5. The cells were incubated for 5 min with various concentrations of shikonin. The cell lysate was mixed with anti-Myc antibody. The immunoprecipitate was analyzed for protein kinase activity with Crosstide as the substrate (C and D) or subjected to Western blotting analysis with the indicated antibodies (E). In D, the cells were pretreated with 1 μM wortmannin (Wort) or 100 μM LY294002 (LY) for 10 min before the addition of 50 μM shikonin.

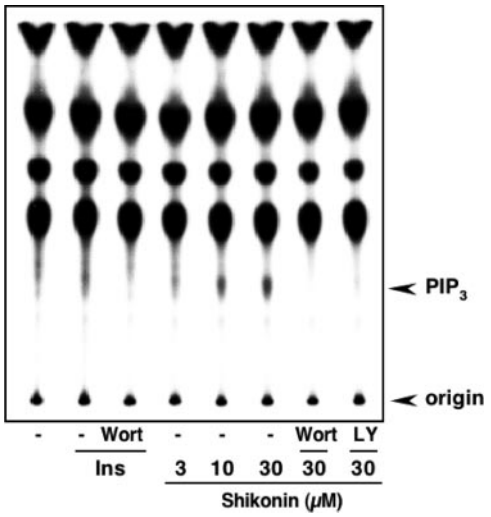

Fig. 2. Effects of shikonin on PtdIns-3,4,5-P_3 production in CHO-IR cells. CHO-IR cells were labeled with $^{32}P_i$. The cells were incubated for 1 min with 100 nM insulin, or for 5 min with the indicated concentrations of shikonin. When the effects of the PI 3-kinase inhibitors were examined, the cells were pretreated with 1 μM wortmannin (Wort) or 100 μM LY294002 (LY) for 10 min before the addition of insulin or shikonin. The lipids were extracted and separated onto thin-layer chromatography plates, and the radioactivity was detected by autoradiography.

phosphatase activity in a dose-dependent manner with an IC_{50} value of 2.7 μM. This observation suggested that the effects of shikonin on intact cells are based on not only inhibition of protein tyrosine phosphatases but also on inhibition of PTEN.

Next, we compared the effect of shikonin with that of vanadate, a well known pan-inhibitor of tyrosine phosphatases. Vanadate inhibited the pNPP phosphatase activities of PTP1B, SHP-1, and Cdc25A at lower concentrations than shikonin (Table 1). These effects may be the mechanism by which pervanadate, an analog of vanadate, caused marked phosphorylation of IRβ and PKB in CHO-IR cells (Fig. 5C). It is interesting to note that the effect of shikonin on PKB was as prominent as that of pervanadate, although its effect on IRβ was far weaker (Fig. 5C). This discrepancy may be explained by the fact that vanadate, unlike shikonin, did not inhibit PTEN at concentrations as high as 100 μM (Table 1).

Many naphthoquinone derivatives have been reported to cause diverse effects on intact cells through their effects on protein tyrosine phosphatases (Ham et al., 1998; Ni et al., 1998; Urbanek et al., 2001). Because shikonin is a derivative of 1,4-naphthoquinone, it is intriguing to consider that several effects of the naphthoquinone derivatives are based on their effect on PTEN. Thus, we examined the effects of two simple naphthoquinone compounds, 1,2-naphthoquinone and 5,8-dihydroxy-1,4-naphthoquinone, on PTEN activity. Their effects on PTP1B, SHP-1, and Cdc25A were also examined. As shown in Fig. 6, these compounds inhibited both PTEN and tyrosine phosphatases, although the effective concentrations were different.

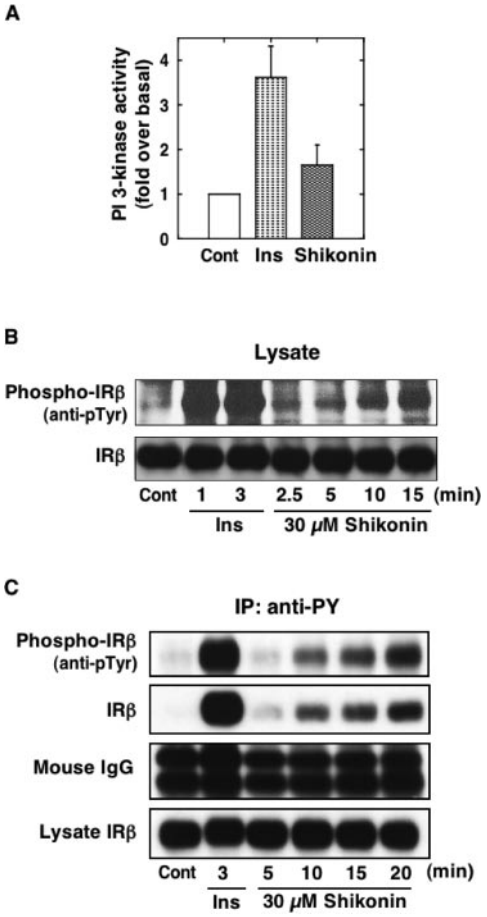

Fig. 3. Effects of shikonin on PI 3-kinase activity and IRβ phosphorylation. A, CHO-IR cells were incubated for 5 min with 100 nM insulin or 30 μM shikonin. The cell lysates were mixed with anti-phosphotyrosine antibody, and the immunoprecipitate was incubated with phosphatidylinositol and [γ-^{32}P]ATP to determine the PI 3-kinase activity. B and C, CHO-IR cells were incubated for the indicated times with 100 nM insulin or 30 μM shikonin. The cell lysates were analyzed by immunoblotting with the indicated antibodies, either directly (B) or after immunoprecipitation with anti-phosphotyrosine antibody (C).

Discussion

Shikonin has been shown to stimulate glucose uptake and GLUT4 translocation in several cell lines (Kamei et al.,

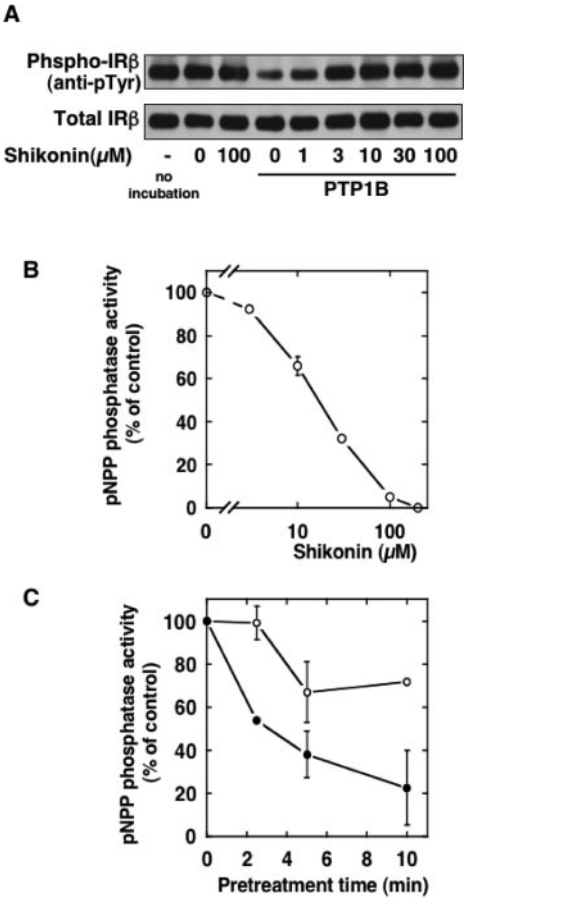

Fig. 4. Effects of shikonin on PTP1B. A and B, His-PTP1B was incubated at 30°C for 10 min with the indicated concentrations of shikonin. In A, the mixture was further incubated for 10 min after the addition of WGA-agarose beads that bound phosphorylated IRβ. The level of tyrosine phosphorylation and the total amount of IRβ were determined by Western blotting analysis. In B, the mixture was further incubated for 10 min after the addition of 1 mM pNPP. The production of *p*-nitrophenol was determined. C, histidine-PTP1B was incubated with 20 μM shikonin for the indicated times in the presence (○) or absence (●) of 5 mM phenylphosphate. The mixture was diluted 100-fold before the determination of pNPP phosphatase activity.

TABLE 1
IC_{50} values of shikonin and vanadate against PTPs and PTEN
IC_{50} values are the means ± S.E. from two to three determinations.

Compound	PTP1B	SHP-1	Cdc25A	PTEN
		μM		
Shikonin	17 ± 1.0	18 ± 1.1	7.0 ± 1.00	2.7 ± 0.86
Vanadate	0.30 ± 0.121	0.62 ± 0.170	0.28 ± 0.060	No inhibition

2002). However, its mechanism of action has not been clarified. The present study suggested that the insulin-like actions of shikonin are due to modulation of PI 3-kinase-dependent signaling. Shikonin induced accumulation of PtdIns-3,4,5-P_3, a product of PI 3-kinase, in intact cells (Fig. 2). The activation of the signaling pathway was confirmed by phosphorylation and activation of PKB (Fig. 1). As possible mechanisms of action, shikonin was found to inhibit both the IRβ phosphatase activity of PTP1B (Fig. 4) and the PtdIns-3,4,5-P_3 phosphatase activity of PTEN (Fig. 5) in cell-free systems.

Insulin signaling is initiated by activation of the tyrosine kinase activity of IRβ and the increased tyrosine phosphorylation of signaling proteins in target cells. Tyrosine phosphatases, including PTP1B, TCPTP, and PTPα, have been reported to dephosphorylate and inactivate IRβ (Moller et al., 1995; Bandyopadhyay et al., 1997; Salmeen et al., 2000; Wälchli et al., 2000; Lacasa et al., 2005). Mice lacking the PTP1B gene show enhanced sensitivity to insulin (Elchebly et al., 1999; Klaman et al., 2000). Insulin-induced phosphorylation of IRβ and activation of PKB/Akt were enhanced in immortalized TCPTP(−/−) murine embryo fibroblasts (Galic et al., 2003). The results of the present study showed that shikonin, like other naphthoquinone compounds, inhibits tyrosine phosphatases in cell-free systems (Table 1). This inhibition may be one mechanism responsible for its insulin-like action. However, the effect of shikonin on phosphorylation of IRβ in intact cells was far weaker than those of insulin and pervanadate, despite the observation that the effect on PKB was as potent as or even more potent than that of pervanadate (Fig. 5). These observations suggest that the increased tyrosine phosphorylation is not the sole mechanism of action of shikonin. In agreement with this speculation, low concentrations of shikonin were reported to augment glucose uptake even when tyrosine phosphorylation has been otherwise increased by pervanadate (Kamei et al., 2002).

An intriguing observation in the present study was the inhibition of PTEN by shikonin (Fig. 5). PTEN is a lipid phosphatase that converts the products of PI 3-kinase to their inactive D-3 dephosphorylated forms. The effect on PTEN explained why shikonin caused marked accumulation of PtdIns-3,4,5-P_3 with minor effects on PI 3-kinase activity or IRβ phosphorylation in intact cells (Fig. 3). PTEN possesses a signature motif, HCXXGXXR, present in the active sites of protein tyrosine phosphatases. The results of crystal

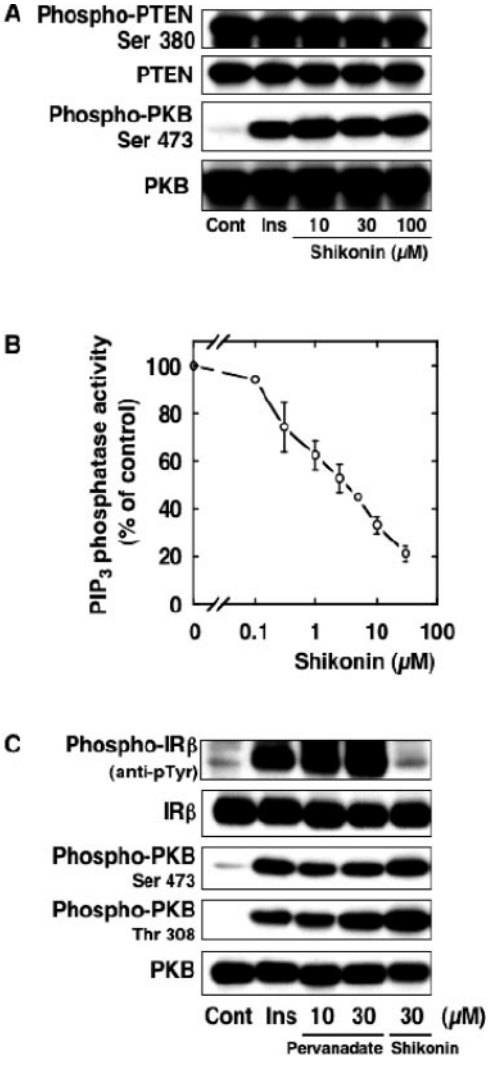

Fig. 5. Effects of shikonin on PTEN. A, CHO-IR cells were incubated with 100 nM insulin or the indicated concentrations of shikonin. The cell lysate was subjected to Western blotting analysis with the indicated antibodies. B, recombinant PTEN was incubated at 37°C for 5 min with various concentrations of shikonin. The mixture was incubated for a further 5 min with the addition of diC16-PtdIns-3,4,5-P_3. The amount of phosphate released was determined by Malachite green assay. C, CHO-IR cells were treated with 30 μM shikonin or indicated concentrations of pervanadate for 5 min or with 100 nM insulin for 3 min. The cell lysates were subjected to Western blotting analysis with the indicated antibodies.

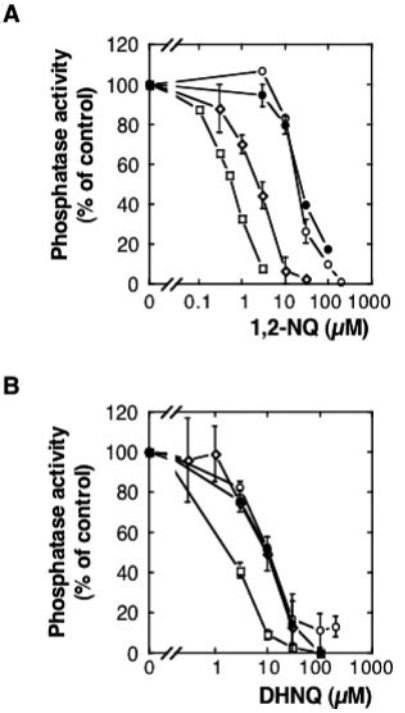

Fig. 6. Effects of naphthoquinone compounds on PTEN and tyrosine phosphatases. Recombinant Cdc25A (◇), His-PTP1B (●), His-SHP1 (○), or PTEN (□) was incubated at 30°C for 10 min with various concentrations of 1,2-naphthoquinone (A) or 5,8-dihydroxyl-1,4-naphthoquinone (B). The activities of tyrosine phosphatases were determined with pNPP as the substrate. The concentrations of pNPP approximated the K_m values of each phosphatase: PTP1B, 1 mM; SHP1, 26 mM; and Cdc25A, 45 mM. The PTEN activity was determined with 0.1 mM diC16-PtdIns-3,4,5-P_3 as the substrate.

structure analysis demonstrated similarity between the active pockets of PTEN and tyrosine phosphatases (Lee et al., 1999). Thus, shikonin may inhibit the enzymes by binding to their active pockets. It has been reported that inhibition of Cdc25 by a naphthoquinone derivative involves an interaction with residues within the active site of the enzyme (Kerns et al., 1995; Ham et al., 1997). The results of the present study showed that shikonin inhibits PTP1B in an irreversible manner, and this inhibition is protected by the substrate of the enzyme (Fig. 4C). Thus, shikonin is considered to inhibit PTEN in a similar manner.

We showed that shikonin is an inhibitor of both PTEN and protein tyrosine phosphatases. Its effect on PTEN provides insight into its potent insulin-like actions as discussed above. PTEN is a tumor suppressor, the mutation or deletion of which is observed in approximately 50% of glioblastoma, endometrial carcinoma, prostate carcinoma, and melanoma cases (Cantley and Neel, 1999). It is also known that DJ1, a genetic negative regulator of PTEN, is associated with breast and lung cancers (Cully et al., 2006). In the present study, we showed that naphthoquinone derivatives, including 1,2-naphthoquinone, inhibit PTEN at concentrations similar to that of shikonin. Many naphthoquinone derivatives have been reported to cause diverse effects in intact cells through the inhibition of protein tyrosine phosphatases (Ham et al., 1998; Ni et al., 1998; Urbanek et al., 2001). Their effects on PTEN and thus PI 3-kinase signaling should be taken into account when the pharmacological actions of the naphthoquinone derivatives are examined.

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