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Halenaquinone, a novel phosphatidylinositol 3-kinase inhibitor from a marine sponge, induces apoptosis in PC12 cells

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Abstract

In nerve growth factor-treated PC12 cells, 12*b*-methyl-(*S*)-1*H*-benzo[6,7]phenanthro[10,1-*bc*]furan-3,6,8,11(2*H*,12*bH*)-tetrone (halenaquinone) caused cytotoxicity in a concentration-dependent manner (EC_{50} value; 10 μM). Gel electrophoretic DNA analysis of PC12 cells treated with halenaquinone (10 μM) and 11-(acetyloxy)-1,6*b*,7,8,9*a*,10,11,11*b*-octahydro-1-(methoxymethyl)-9*a*,11*b*-dimethyl-[1*S*-(1α,6*b*α,9*a*β,11α,11*b*β)]-3*H*-furo[4,3,2-*de*]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione (wortmannin) (3 μM) showed a typical apoptotic DNA ladder. In the flow cytometric analysis, halenaquinone caused apoptosis in a concentration- and time-dependent manner (EC_{50} value; 10 μM), whereas 2,3-dihydro-12*b*-methyl-(*S*)-1*H*-benzo[6,7]phenanthro[10,1-*bc*]furan-6,8,11(12*bH*)-trione (xestoquinone) with the methylene group at the C-3 position failed to cause apoptosis, suggesting that the carbonyl group at the C-3 position in halenaquinone is important for exerting apoptotic effects in PC12 cells. Phosphatidylinositol 3-kinase was inhibited by halenaquinone (IC_{50} value; 3 μM) as well as wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. Halenaquinone inhibited phosphatidylinositol 3-kinase activity at lower concentrations than those at which it induced apoptosis in PC12 cells. These results suggest that halenaquinone causes the death of PC12 cells through an apoptotic process and that the mechanism of halenaquinone-induced apoptosis may be partially explained by the inhibition of phosphatidylinositol 3-kinase activity.

Keywords: Halenaquinone; Apoptosis; Phosphatidylinositol 3-kinase inhibitor; PC12 cell; Pharmacological tool

1. Introduction

Apoptosis is a programmed cell death process and plays an important role in the survival and development of an organism. Apoptosis can be prevented in many instances by growth factors (Yao and Cooper, 1995; Len and Wong, 1999). It has been reported that inhibitors of apoptosis, such as (−)-(*R*)-2-[4-[[(3,4-dihydro-2*H*-1-benzopyran-2-yl) methyl] amino] butyl]-1,2-benzisothiazol-3(2*H*)-one-1,1-dioxide monohydrochloride (BAY × 3702), ginkgolide B and bilobalide, are very useful pharmacological tools (Suchanek et al., 1998; Ahlemeyer et al., 1999). In several different types of neurons and neuronal cells, nerve growth factor (NGF) activates the TrkA tyrosine kinase receptor (Clary et al., 1993; Rovelli et al., 1993). Then TrkA autophosphorylates specific tyrosine residues within its intracellular domain and the phosphorylated tyrosines serve as protein interaction sites for phosphatidylinositol 3-kinase (Bartlett et al., 1997). Activated phosphatidylinositol 3-kinase increases the activity of Akt, which acts by phosphorylating Bcl-x_L/Bcl-2-associated death promoter (BAD), a member of the Bcl-2 family of proteins (Datta et al., 1997). Consequently, NGF can mediate phosphatidylinositol 3-kinase activation and suppress apoptosis in a

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number of cell lines. Yao and Cooper (1995) reported that in PC12 cells the inhibition of phosphatidylinositol 3-kinase activity by selective phosphatidylinositol 3-kinase inhibitors, such as 11-(acetyloxy)-1,6*b*,7,8,9*a*,10,11,11*b*-octahydro-1-(methoxymethyl)-9*a*,11*b*-dimethyl-[1*S*-(1α,6*b*α,9*a*β,11α,11*b*β)]-3*H*-furo[4,3,2-*de*]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione (wortmannin) (Fig. 1C) and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), induced apoptosis even in the presence of NGF (Yao and Cooper, 1995). Moreover, Wymann et al. suggested that wortmannin inhibited phosphatidylinositol 3-kinase by interacting with the p110α catalytic subunit of phosphatidylinositol 3-kinase (Wymann et al., 1996).

Numerous marine natural products have been used as pharmacological tools for pharmacological, physiological and biochemical studies (Ohizumi, 1997). Recently, we have found that several marine natural products such as goniodomin A (Furukawa et al., 1993), purealin (Takito et al., 1986; Nakamura et al., 1987) and 2,3-dihydro-12*b*-methyl-(*S*)-1*H*-benzo[6,7]phenanthro[10,1-*bc*]furan-6,8,11(12*bH*)-trione (xestoquinone) (Fig. 1B) (Sakamoto et al., 1995) modulate actomyosin ATPase activity. In the course of our survey of pharmacological tools from marine sources, much attention has been given to compounds inducing apoptosis because of their important role in studies of signal transduction (Kugawa et al., 1998). More recently, 12*b*-methyl-(*S*)-1*H*-benzo[6,7]phenanthro[10,1-*bc*]furan-3,6,8,11(2*H*,12*bH*)-tetrone (halenaquinone) (Fig. 1A) has been isolated from the Okinawan sea sponge *Xestospongia exigua* and has been found to induce apoptosis in PC12 cells. Xestoquinone is structurally analogous to halenaquinone except it has a methylene group at the C-3 position. Halenaquinone has been reported to have antibiotic activity (Roll et al., 1983), antiproliferative activity (Schmitz and Bloor, 1988) and cytotoxic activity against L1210 cells (Kobayashi et al., 1992). However, the detailed biochemical and pharmacological properties of this compound have not been reported yet. Here, we present the first paper on apoptosis induced by halenaquinone. This compound has become a useful pharmacological tool for studying the molecular mechanism of apoptosis.

2. Materials and methods

2.1. Materials

Halenaquinone and xestoquinone were isolated from the Okinawan sea sponge *X. exigua* and *X. sapra* as previously reported, respectively (Roll et al., 1983; Nakamura et al., 1985). The grade of purity of halenaquinone was confirmed on the basis of nuclear magnetic resonance data (> 99% purity). For all experiments, halenaquinone, xestoquinone and wortmannin were dissolved in dimethyl sulfoxide; the concentration of dimethyl sulfoxide was 0.1%.

Wortmannin, 7S NGF and RNase A were obtained from Sigma (St. Louis, MO, USA). Fetal calf serum and horse serum were from Cell Culture Laboratory (Cleveland, OH, USA) and ICN Biochemicals (Costa Mesa, CA, USA), respectively. RPMI 1640 was obtained from Nissui Pharmaceuticals (Tokyo, Japan). Anti-rat phosphatidylinositol 3-kinase antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Phospho-Akt antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit immunoglobulin G (IgG) antibody alkaline phosphatase-linked and anti-mouse IgG antibody alkaline phosphatase-conjugate were from Bio-Rad (Hercules, CA, USA). Protein A Sepharose suspension was obtained from ZYMED Laboratories (San Francisco, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Dojindo (Kumamoto, Japan). [γ-^{32}P]ATP (10 mCi/ml) and [α-^{32}P]dCTP (10 mCi/ml) were from Amersham International (Buckinghamshire, England). Klenow fragment was from TaKaRa shuzo (Shiga, Japan). All other reagents or drugs were of analytical grade.

2.2. Cell culture

PC12 cells were maintained in RPMI 1640 supplemented with 5% fetal calf serum, 10% heat-inactivated horse serum and 4 mM L-glutamine in a humidified atmo-

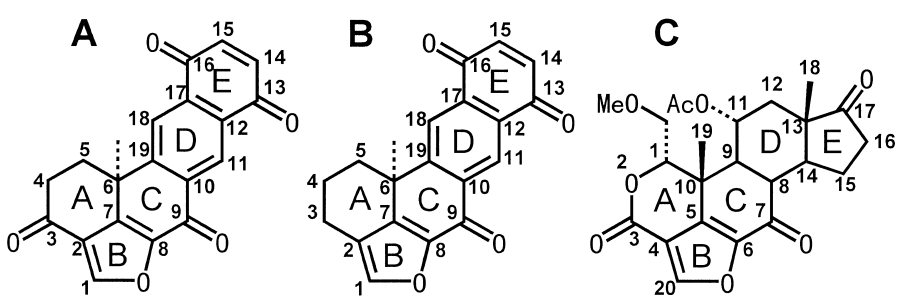

Fig. 1. Chemical structures of halenaquinone (A), xestoquinone (B) and wortmannin (C).

sphere of 5% CO_2 and 95% air, as previously reported (Obara et al., 1998).

2.3. Cell viability assay

Cell viability was assessed using the MTT method as previously reported (Taglialatela et al., 1997) with a slight modification. PC12 cells were seeded in 96-well plates at a density of 1×10^4 cells per well in the presence of NGF with or without drugs. After 24 h, plates were read on the microplate reader (Model 450, Bio-Rad) at a test wavelength of 595 nm.

2.4. Morphological observation by using fluorescence microscopy

PC12 cells were seeded in 24-well plates at a density of 5×10^4 cells per well. After 24 h, medium was switched to a 100 ng/ml NGF-containing serum-free RPMI 1640. Cells were treated with 10 μM halenaquinone and 3 μM wortmannin in 100 ng/ml NGF-containing serum-free RPMI 1640 for 24 h. Cells were stained with 50 ng/ml propidium iodide for 30 min. PC12 cells were rinsed with phosphate-buffered saline (PBS) and visualized by fluorescence microscopy (IMT-2, Olympus, Tokyo, Japan).

2.5. DNA analysis

DNA analysis was carried out by the method of Kobayashi et al. (1994) with slight modifications. PC12 cells were seeded in 100-mm dishes at a density of 1×10^6 cells per dish. After 24 h, medium was switched to 100 ng/ml NGF-containing serum-free RPMI 1640. Cells were treated with 10 μM halenaquinone and 3 μM wortmannin in 100 ng/ml NGF-containing serum-free RPMI 1640 for 24 h. Cells were resuspended in cell lysis buffer (20 mM 2-amino-2-hydroxymethyl-1, 3-propanediol (Tris)–HCl (pH 7.4), 5 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) (pH 8.0) and 0.1% polyoxyethylene (10) octylphenyl ether (Triton X-100)). The soluble DNA in the supernatant after centrifugation at $15{,}000 \times g$ for 20 min was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol at −20°C for 1 h and resuspended in Tris–EDTA buffer (10 mM Tris–HCl (pH 7.4) and 1 mM EDTA (pH 8.0)). DNA was incubated with 10 μg/ml RNase A at 37°C for 1 h and then labeled with 0.1 mCi/ml [α-^{32}P]dCTP using 5 units of Klenow fragment for 10 min, followed by re-precipitation on ethanol at −20°C for 1 h. Electrophoresis was carried out on a 2% agarose gel at 50 V for 90 min in Tris–borate–EDTA buffer (89 mM Tris, 89 mM borate and 2 mM EDTA (pH 8.0)). The gel was dried and treated with the molecular imager (G363, Bio-Rad).

2.6. Apoptotic analysis by flow cytometry

Apoptotic cells were detected according to the method of Darzynkiewicz et al. (1992) with a slight modification. PC12 cells were seeded in 6-well plates at a density of 1×10^5 cells per well. After 24 h, medium was switched to 100 ng/ml NGF-containing serum-free RPMI 1640. Cells were treated with halenaquinone in 100 ng/ml NGF-containing serum-free RPMI 1640 for 24 h. Cells were fixed with 70% ethanol at −20°C for 2 h. Cells were rinsed with PBS, incubated with 50 μg/ml RNase A at 37°C for 15 min and stained with 50 μg/ml propidium iodide for 10 min. Apoptotic analysis was performed by using a flow cytometer (FACScan, Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA).

2.7. Phosphatidylinositol 3-kinase immunoprecipitation

Phosphatidylinositol 3-kinase was immunoprecipitated according to the method of Kimura et al. (1994) with a slight modification. Cells were lysed with Nonidet P-40 buffer (50 mM Tris–HCl (pH7.4), 20 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride and 1 mM sodium orthovanadate). Lysates were clarified at $10{,}400 \times g$ at 4°C for 20 min and incubated with anti-rat phosphatidylinositol 3-kinase at 4°C overnight. Protein A Sepharose suspension (two volumes) was added to the lysates and the

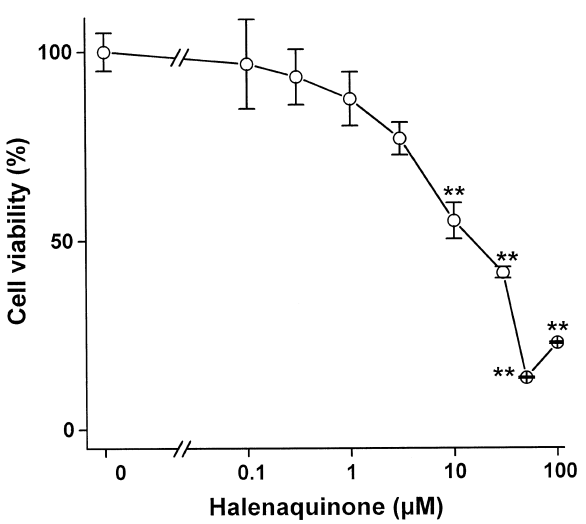

Fig. 2. Effects of halenaquinone on the viability of NGF-treated PC12 cells. Cell viability was assessed by the MTT method as described in Materials and methods. PC12 cells were treated with several concentrations of halenaquinone for 24 h. Cell viability is expressed as a percentage of the control viability (100%) in the absence of halenaquinone. Values represent the means ± S.E.M. from four experiments. Statistically significant difference from the control viability is indicated in the figure: ** $P < 0.01$.

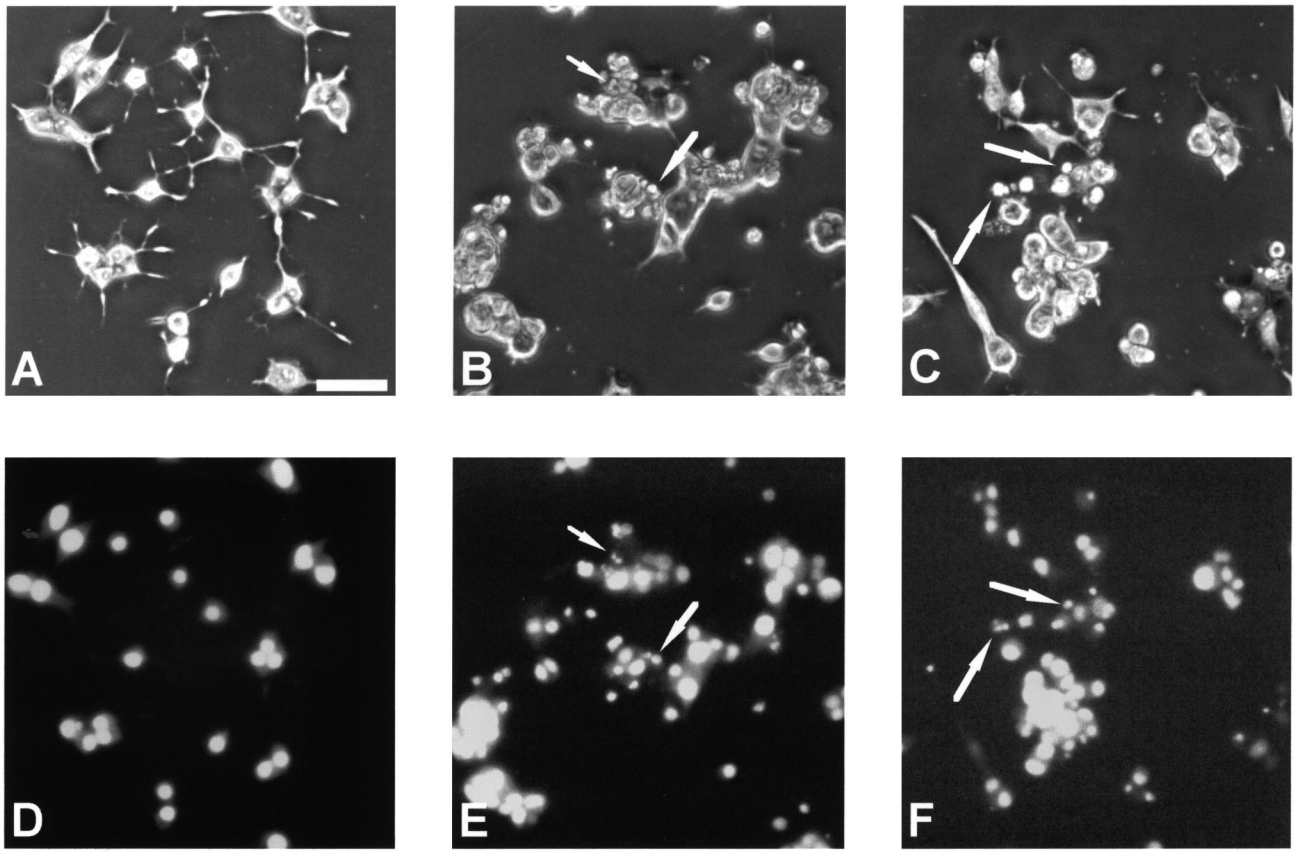

Fig. 3. Morphological changes in PC12 cells. Phase-contrast photomicrographs show cell morphology (A–C) after propidium iodide-staining of chromatin (D–F). Control (A, D), 10 μM halenaquinone (B, E) and 3 μM wortmannin (C, F). Arrowheads point to a representative apoptotic cell with a degraded soma. Scale bar, 50 μm.

mixed suspension was incubated for 2 h at 4°C. The immune complexes were centrifuged at 1,500 × *g* at 4°C for 2 min and washed three times with Nonidet P-40 buffer.

2.8. Phosphatidylinositol 3-kinase activity

Immunoprecipitates of phosphatidylinositol 3-kinase were suspended with PBS and treated with several concentrations of halenaquinone at 30°C for 10 min. The immunoprecipitate solution (30 μl) was incubated with 50 μl of assay mixture (0.2 mM phosphatidylinositol, 0.375 mM phosphatidylserine, 60 mM Tris–HCl (pH 7.4) and 5 mM ethyleneglycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) (pH 8.0)). The reaction was initiated by the addition of 20 μl of ATP mixture (25 mM $MgCl_2$, 0.5 mM ATP and 0.2 mCi/ml [γ-^{32}P]ATP), allowed to proceed at 30°C for 30 min and stopped by 470 μl of the stop solution (methanol:chloroform:6% perchloric acid (35:15:2, v/v)). After 10 min, 150 μl of chloroform, 75 μl of 2% perchloric acid and 75 μl of 2 M NaCl were added to the reaction mixture. It was mixed and centrifuged for 5 min at room temperature. The lower chloroform phase was collected and applied to the thin-layer

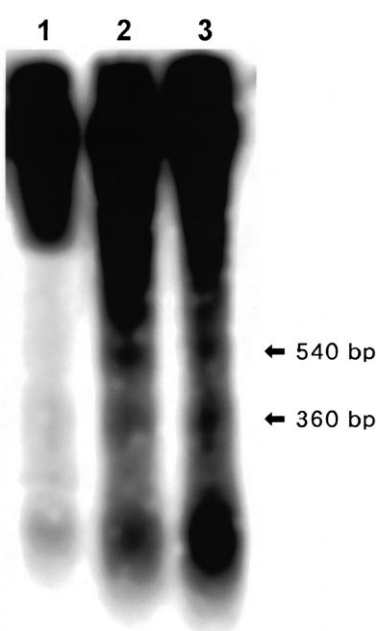

Fig. 4. DNA fragmentation of PC12 cells. PC12 cells were treated with 10 μM halenaquinone (lane 2) and 3 μM wortmannin (lane 3) for 24 h. Bands of 360 and 540 bp can be seen after 2% agarose gel electrophoresis whereas DNA from untreated cells (lane 1) remains intact.

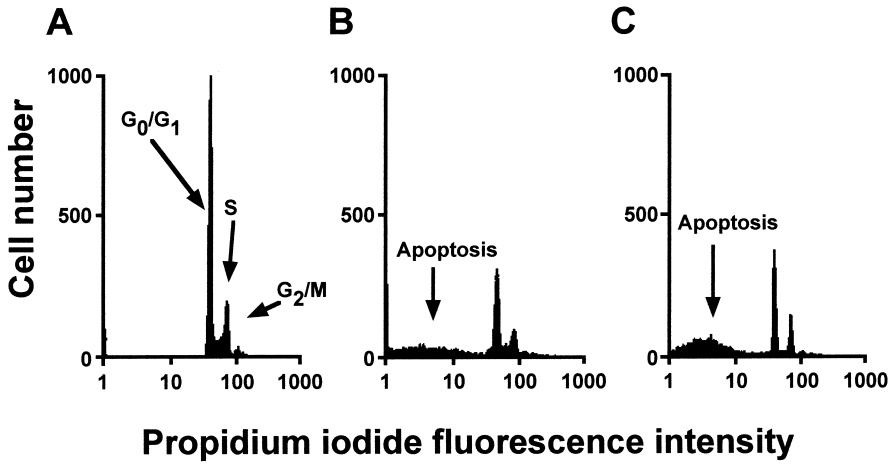

Fig. 5. Typical histograms of the flow cytometric analysis of halenaquinone-treated PC12 cells. Apoptosis was measured by flow cytometry as described in Materials and methods. Each histogram shows the number of untreated (A), 10 μM halenaquinone-treated (B), and 3 μM wortmannin-treated (C) cells as a function of propidium iodide fluorescence. Arrowheads point to peak of each cell cycle. Apoptotic cells are defined as cells with a low DNA content.

chromatography (TLC) plate. The TLC was run in a tank equilibrated with chloroform:acetone:methanol:acetic acid:water (40:15:13:12:7, v/v). The radioactivity of [^{32}P]-phosphatidylinositol monophosphate on the TLC plate was detected with the molecular imager.

2.9. Western blotting

Western blotting was performed according to the method of Kimura et al. (1994) with a slight modification. Cells were lysed with Nonidet P-40 buffer. Lysates were heated in sodium dodecyl sulfate (SDS) sample buffer (50% glycerol, 4% SDS, 25 mM Tris (pH 6.8), 5% mercaptoethanol and 0.01% Coomassie brilliant blue), electrophoresed on a 10% polyacrylamide gel and transferred onto a polyvinylidenedifluoride membrane. After incubation with Tris-buffered saline (10 mM Tris–HCl (pH 7.4) and 100 mM NaCl) containing 0.05% Tween 20 and 1% bovine serum albumin for 2 h, the membrane was treated with anti-phosphotyrosine or phospho-Akt antibody at 4°C overnight, followed by incubation with anti-mouse IgG antibody alkaline phosphatase-conjugate or anti-rabbit IgG antibody alkaline phosphatase-linked for 2 h. The bands were detected with the ECL system.

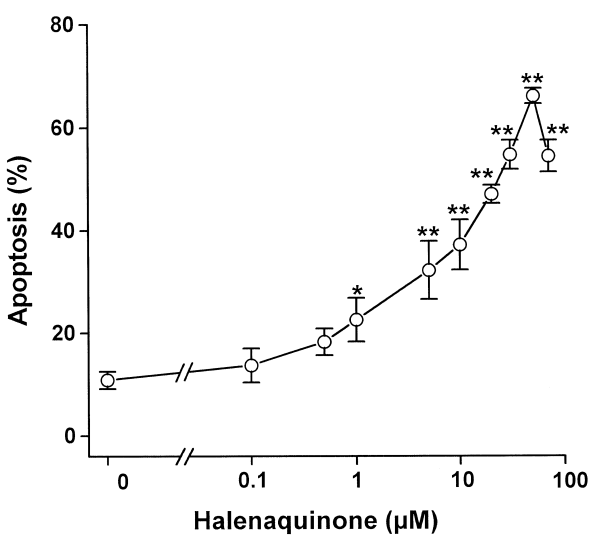

Fig. 6. Concentration-dependent increase in apoptosis induced by halenaquinone in PC12 cells. PC12 cells were treated with several concentrations of halenaquinone for 24 h. Apoptosis is expressed as a percentage of the number of apoptotic cells to the total number of cells. Values represent the means ± S.E.M. from three experiments. Statistically significant difference from the control in the absence of halenaquinone is indicated in the figure: $^{*}P < 0.05$, $^{**}P < 0.01$.

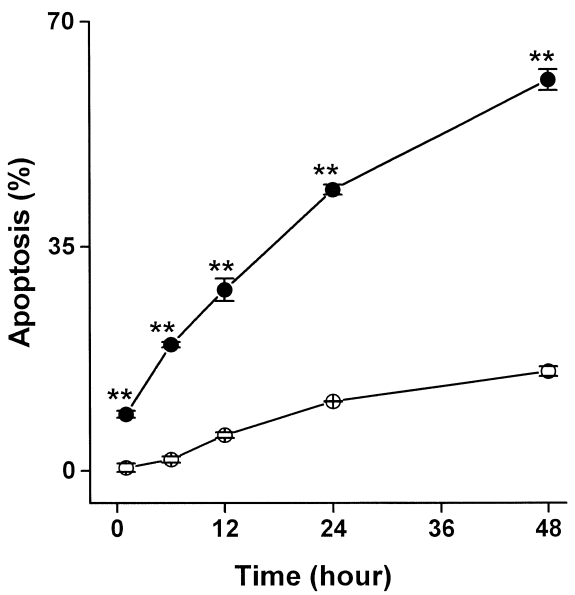

Fig. 7. Time-dependent increase in apoptosis in the presence (●) or absence (○) of 10 μM halenaquinone in PC12 cells. Apoptosis is expressed as a percentage of the number of apoptotic cells to the total number of cells. Values represent the means ± S.E.M. from three experiments. Statistically significant difference from the control in the absence of halenaquinone is indicated in the figure: $^{**}P < 0.01$.

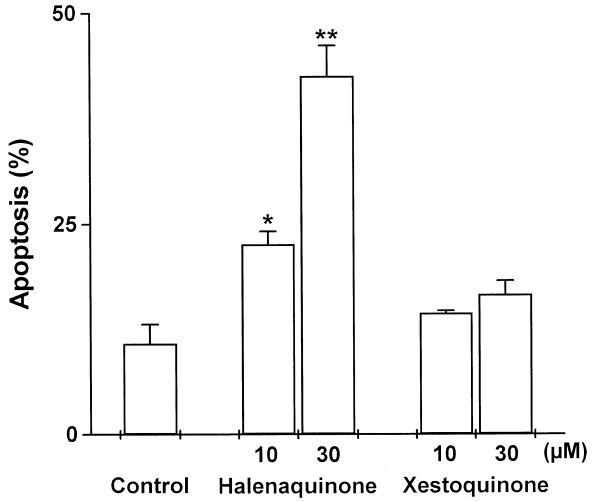

Fig. 8. Apoptotic effects of halenaquinone and xestoquinone in PC12 cells. PC12 cells were treated with halenaquinone or xestoquinone for 24 h. Apoptosis is expressed as a percentage of the number of apoptotic cells to the total number of cells. Values represent the means ± S.E.M. from three experiments. Statistically significant difference from the control is indicated in the figure: $^{*}P < 0.05$, $^{**}P < 0.01$.

2.10. Statistical analysis of the data

The data are expressed as means ± S.E.M. Statistical comparisons were made by using Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Cell viability of halenaquinone-treated PC12 cells

The effects of halenaquinone on the viability of NGF-treated PC12 cells were examined. Halenaquinone in the range of 0.1 to 50 μM induced cell death in a concentration-dependent manner with a 50% effective concentration (EC_{50}) of 10 μM (Fig. 2).

3.2. Apoptotic morphological changes and DNA fragmentation in halenaquinone-treated PC12 cells

Halenaquinone at a concentration of 10 μM shrank cell soma and fragmented neurites (Fig. 3B). Halenaquinone-treated PC12 cells contained one or more compact spheres of condensed chromatin in their nuclei (Fig. 3E), in contrast to the chromatin uniformly dispersed in the nuclei of untreated PC12 cells (Fig. 3A and D). These characteristics of halenaquinone-treated cells were similar to those of 3 μM wortmannin-treated cells (Fig. 3C and F). To characterize the mode of cell death more clearly, it was examined whether typical apoptotic DNA fragmentation was induced by halenaquinone in PC12 cells. DNA isolated from 10 μM halenaquinone- and 3 μM wortmannin-treated cells showed typical apoptotic DNA ladders (bands of 360 and 540 bp, etc., Fig. 4, lane 2 and lane 3). DNA

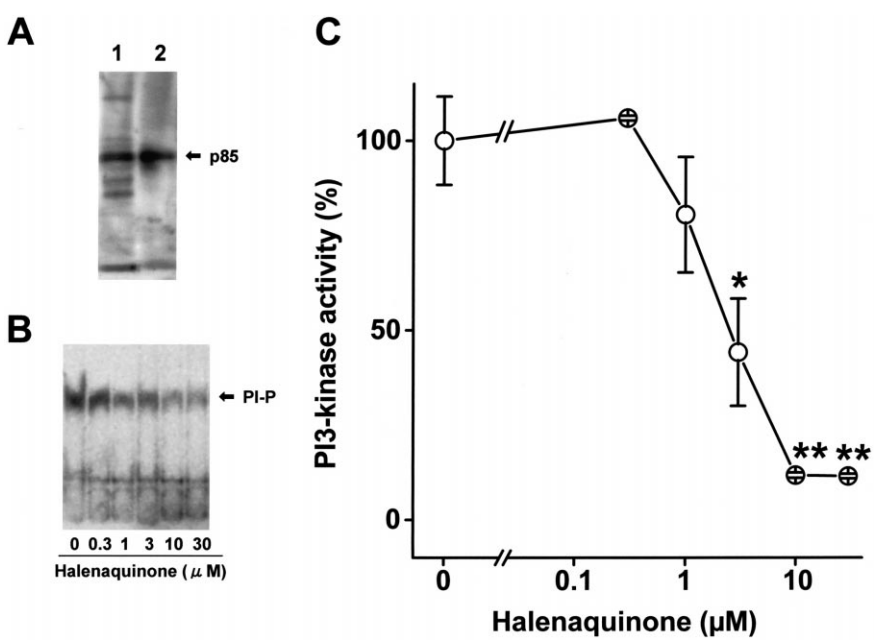

Fig. 9. Immunoprecipitation of phosphatidylinositol 3-kinase and inhibitory effects of halenaquinone on phosphatidylinositol 3-kinase activity. (A) Western blotting analysis of phosphatidylinositol 3-kinase with (lane 2) or without (lane 1) immunoprecipitation. Phosphatidylinositol 3-kinase was immunoprecipitated from PC12 cells with anti-rat phosphatidylinositol 3-kinase p85 subunit antibody and detected with anti-phosphotyrosine antibody. (B) The TLC analysis of [^{32}P]phosphatidylinositol monophosphate (PI-P) and (C) the measurement of the density of the spot corresponding to PI-P. Phosphatidylinositol 3-kinase was incubated with several concentrations of halenaquinone at 30°C for 10 min. The phosphatidylinositol 3-kinase activity is expressed as a percentage of control activity (100%) in the absence of halenaquinone. Values represent the means ± S.E.M. from three experiments. Statistically significant difference from the control activity is indicated in the figure: $^{*}P < 0.05$, $^{**}P < 0.01$.

from untreated cells did not show a DNA ladder (Fig. 4, lane 1).

3.3. Flow cytometric analysis of apoptosis in halenaquinone-treated PC12 cells

Fig. 5 shows typical histograms of the flow cytometric analysis of apoptotic cell death in PC12 cells in the presence or absence of 10 μM halenaquinone or 3 μM wortmannin. Halenaquinone caused concentration-dependent apoptosis in the range of 0.1 to 50 μM with an EC_{50} of 10 μM (Fig. 6). Apoptosis was induced by halenaquinone within 6 h and reached about 60% after 48 h (Fig. 7).

3.4. Apoptotic effects of xestoquinone

Xestoquinone, which is analogous to halenaquinone (Fig. 1B), did not cause apoptosis in PC12 cells even at concentrations of 30 μM (Fig. 8) and 100 μM (data not shown).

3.5. Inhibition of phosphatidylinositol 3-kinase activity by halenaquinone

Phosphatidylinositol 3-kinase was prepared from PC12 cells by immunoprecipitation and its identity was confirmed by Western blotting (Fig. 9A). Halenaquinone at concentrations of 1, 3, 10 and 30 μM inhibited phosphatidylinositol 3-kinase activity in a concentration-dependent manner, with a 50% inhibitory concentration (IC_{50}) of 3 μM (Fig. 9B and C). Phosphatidylinositol 3-kinase activity was inhibited by wortmannin (3 μM) by about 70% (data not shown). The activity of phosphatidylinositol 3-kinase was abolished by pretreatment of PC12 cells with 10 μM halenaquinone and 3 μM wortmannin (data not shown). In addition, xestoquinone did not affect phosphatidylinositol 3-kinase activity even at a concentration of 30 μM (data not shown).

3.6. Inhibition of Akt phosphorylation by halenaquinone

In PC12 cells, Akt, an effector of phosphatidylinositol 3-kinase, was phosphorylated with 50 ng/ml NGF (Fig. 10, lane 1). Halenaquinone at 10 μM and wortmannin at 3 μM blocked NGF-stimulated Akt phosphorylation (Fig. 10, lane 2 and lane 3).

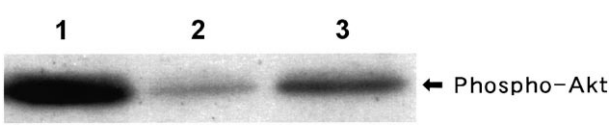

Fig. 10. Blocking effects of halenaquinone on Akt phosphorylation. Akt phosphorylation was assessed by Western blotting as described in Materials and methods. Lane 1, untreated PC12 cells; lane 2, 10 μM halenaquinone-treated cells; lane 3, 3 μM wortmannin-treated cells. The samples were fractionated on a 10% polyacrylamide gel, followed by immunoblotting with phospho-Akt antibody.

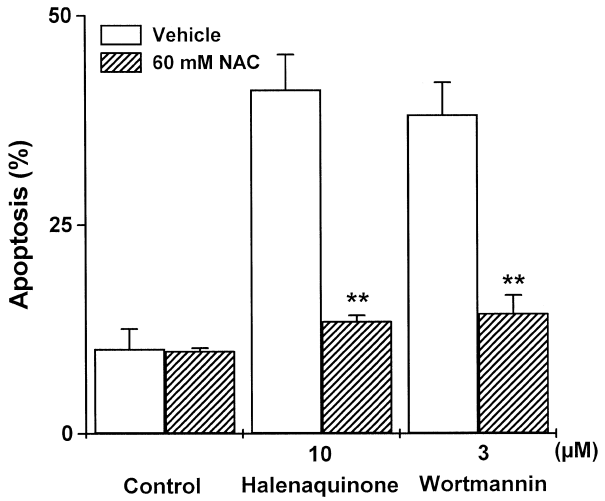

Fig. 11. Effects of *N*-acetyl-L-cysteine on halenaquinone- and wortmannin-induced apoptosis in PC12 cells. PC12 cells were treated with 10 μM halenaquinone or 3 μM wortmannin in the presence or absence of 60 mM *N*-acetyl-L-cysteine for 24 h. Apoptosis is expressed as a percentage of the number of apoptotic cells to the total number of cells. Values represent the means ± S.E.M. from three experiments. Statistically significant difference from the vehicle-treated cells is indicated in the figure: $^{**}P < 0.01$.

3.7. Effects of N-acetyl-L-cysteine on halenaquinone- and wortmannin-induced apoptosis

N-acetyl-L-cysteine at 60 mM prevented 10 μM halenaquinone- or 3 μM wortmannin-induced apoptosis (Fig. 11).

4. Discussion

It has been reported that withdrawal of serum causes apoptotic cell death in PC12 cells (Batistatou and Greene, 1993; Satoh et al., 1995). NGF prevents the death of PC12 cells even in a serum-free medium and induces neuronal differentiation (Alema et al., 1985; Cunningham et al., 1991). It is well known that NGF is an activator of several intracellular molecules such as phosphatidylinositol 3-kinase, Src, phospholipase C-γ and so on (Kim et al., 1991; Maher, 1988). Phosphatidylinositol 3-kinase mediates a variety of cellular responses by generating 3-phosphoinositides that function directly as second messengers to activate signaling molecules. Resent studies have suggested that apoptosis is induced by the inhibition of phosphatidylinositol 3-kinase activity in several cell lines (Bartlett et al., 1997). Phosphatidylinositol 3-kinase has an important part in the apoptotic mechanism, and apoptosis is induced by a selective phosphatidylinositol 3-kinase

inhibitor, wortmannin, even in the presence of NGF (Yao and Cooper, 1995).

In our present experiments, halenaquinone as well as wortmannin induced cell death with characteristic features of apoptosis such as shrinkage of cell soma, fragmentation of neurites and chromatin condensation. Moreover, halenaquinone caused oligonucleosomal DNA fragmentation which was mediated by DFF40/CAD (Inohara et al., 1999). DNA fragmentation by halenaquinone was further investigated by flow cytometric analysis. Halenaquinone increased the number of cells that contained a small amount of DNA. These results suggest that halenaquinone caused the death of PC12 cells through an apoptotic mechanism.

Halenaquinone and wortmannin possess a similar partial structure in an A–B–C ring system including the carbonyl group at the C-3 position. Xestoquinone does not have the carbonyl group at the C-3 position. An interesting observation is that the reduction of the carbon atom at the C-3 position (halenaquinone → xestoquinone) resulted in a marked loss of apoptotic activity and phosphatidylinositol 3-kinase activity. These observations suggest that the carbonyl group at the C-3 position of halenaquinone participates in the development of apoptotic activity and phosphatidylinositol 3-kinase activity in PC12 cells. It is well known that the carbonyl group is reduced in the metabolic pathway. Studies of the metabolism of halenaquinone are now underway.

Wortmannin induces apoptosis by inhibiting phosphatidylinositol 3-kinase activity (Yao and Cooper, 1995). It has been proposed that wortmannin conjugates with the lysine of phosphatidylinositol 3-kinase at the C-20 position, resulting in the irreversible inhibition of phosphatidylinositol 3-kinase (Wymann et al., 1996). It has been reported that, in PC12 cells, wortmannin exerts apoptotic effects and inhibitory effects on phosphatidylinositol 3-kinase activity (around 150 nM) (Yao and Cooper, 1995). In the present experiment using PC12 cells, wortmannin induced both effects, but required a higher concentration (3 μM). Halenaquinone induced apoptosis (EC_{50} value; 10 μM) and also inhibited phosphatidylinositol 3-kinase activity (IC_{50} value; 3 μM) in a concentration-dependent manner. The activity of phosphatidylinositol 3-kinase immunoprecipitated with halenaquinone or wortmannin disappeared, suggesting that halenaquinone is an irreversible inhibitor as well as wortmannin. Akt, a serine threonine kinase, is activated by phosphatidylinositol 3-kinase. Halenaquinone and wortmannin inhibited Akt phosphorylation. It is a noteworthy observation that the concentration of halenaquinone needed to inhibit phosphatidylinositol 3-kinase activity was lower than that needed to induce apoptosis, suggesting that the inhibition of phosphatidylinositol 3-kinase activity may be at least partially involved in the mechanism of halenaquinone-induced apoptosis in PC12 cells. However, we cannot eliminate the possible involvement of other kinases in addition to phosphatidylinositol 3-kinase in halenaquinone-induced apoptosis.

N-acetyl-L-cysteine has an antioxidant effect (Aruoma et al., 1989). Halenaquinone has a potentially redox active structure. Halenaquinone- or wortmannin-induced apoptosis was completely blocked by *N*-acetyl-L-cysteine. However, xestoquinone, a potentially redox active structure like halenaquinone, did not cause apoptosis in PC12 cells. Further detailed study of the involvement of oxidative stress in the mechanism of halenaquinone-induced apoptosis is now underway.

In conclusion, these results suggest that halenaquinone, like wortmannin, causes the inhibition of phosphatidylinositol 3-kinase activity and thus induces apoptosis in PC12 cells. Halenaquinone may be a valuable pharmacological tool for clarifying the apoptotic mechanism.

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