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# Inositol hexakisphosphate blocks tumor cell growth by activating apoptotic machinery as well as by inhibiting the Akt/NFκB-mediated cell survival pathway

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**It has been reported that inositol hexakisphosphate ($InsP_6$, phytic acid), a natural product, has an anticancer role. However, there is inadequate information regarding the mechanism by which $InsP_6$ exerts anticancer actions, and the effect requires relatively high concentration of the agent, both of which hinders the usage of $InsP_6$ as an anticancer drug. In the present study, we investigated the mechanism by which $InsP_6$ acts as an anticancer agent, and tried to reduce the concentration of effective $InsP_6$. Treatment of HeLa cells with $InsP_6$ at 1 mM induced apoptosis, as assessed by counting the cell number, and by Hoechst and TUNEL staining. This is probably mediated by intracellular $InsP_6$ itself and/or the dephosphorylated forms of metabolized $InsP_6$, because incubation of HeLa cells with [$^3$H]$InsP_6$ produces dephosphorylated forms such as $InsP_4$ and $InsP_5$. Induction of apoptosis by $InsP_6$ was examined in two ways: inhibition of cell survival signaling and direct induction of apoptosis. Treatment of HeLa cells with tumor necrosis factor (TNF) or insulin stimulated the Akt-nuclear factor κB (NFκB) pathway, a cell survival signal, which involves the phosphorylation of Akt and IκB, nuclear translocation of NFκB and NFκB-luciferase transcription activity. $InsP_6$ blocked all these cellular events, but phosphatidylinositol 3-kinase activity was not affected. As well as inhibiting the Akt-NFκB pathway, $InsP_6$ itself caused mitochondrial permeabilization, followed by cytochrome c release, which later caused activation of the apoptotic machinery, caspase 9, caspase 3 and poly (ADP-ribose) polymerase. When $InsP_6$ was applied together with histone, the effective concentration to induce apoptosis was ~10-fold lower. These results revealed that extracellularly applied $InsP_6$ directly activates the apoptotic machinery as well as inhibits the cell survival signaling, probably by the intracellular delivery followed by a dephosphorylation.**

## Introduction

Inositol hexakisphosphate ($InsP_6$) is present in plants, particularly in cereals and legumes, at concentrations ranging from 0.4 to 6.4%. In plants, $InsP_6$ exists as a salt with monovalent and divalent cations such as $Ca^{2+}$, $Mg^{2+}$ and $K^+$ (1,2). Inositol, $InsP_6$ and lower inositol phosphates ($InsP_{1–5}$) are all present as intracellular molecules in mammalian cells as well (1,2). $InsP_5$ and $InsP_6$ are present in virtually all mammalian cells at substantial amounts, between 10 and 100 μM, much higher than any other InsPs (1,2). There are several reports that extracellularly applied $InsP_6$ exhibits an anticancer effect on several cancers in the blood, colon, liver, lung, mammary and prostate (3–8). Comparison of synthetic compounds and natural products, such as $InsP_6$, suggests that the latter may be more suitable for chemoprevention of carcinogenesis, because their actions are milder (9). It has also been reported that $InsP_6$ is involved in cell apoptosis and differentiation (10). As to the mechanisms by which $InsP_6$ exerts anticancer effects, there have been several reports that $InsP_6$ inhibits the transforming growth factor α-stimulated-phosphatidylinositol 3-kinase (PI3K)-Akt pathway in prostate carcinoma cells; $InsP_6$ inhibited PI3K activity, followed by the activation of Akt which is involved in cell survival through the phosphorylation of Bad, a pro-apoptotic molecule, of caspase 9 and/or of IκB kinaseα (IKKα) (11). However, another report is conflicting; $InsP_6$ had no effect on PI3K activation, but was effective in the inhibition of nuclear factor κB (NFκB) activation (12). Since the Akt–NFκB pathway is known to be a strong cell survival pathway (13,14), it is important to determine which step is impaired by $InsP_6$.

The dephosphorylation of $InsP_6$ to $InsP_{1–5}$ could enhance its anticancer function (15). Razzini *et al.* (16) reported that Ins(1,3,4,5,6)$P_5$ and Ins(1,4,5,6)$P_4$, at relatively low concentrations, exhibited anticancer activity in human breast cancer and human ovarian cancer cells to the same extent as $InsP_6$ at millimolar concentrations. This effect is a consequence of the competition between InsPs and PtdIns(3,4,5)$P_3$ for proteins bearing the pleckstrin homology (PH) domain favoring PtdIns(3,4,5)$P_3$, a product of PI3K activity such as that of Akt.

In the present study, we investigated the mechanism of $InsP_6$ internalization and its competence to inhibit cell proliferation and to induce apoptosis. We also looked into the mechanisms by which $InsP_6$ inhibits the tumor necrosis factor (TNF)-induced PI3K–NFκB survival pathway on which we reported previously (14) and whether $InsP_6$ itself causes activation of the apoptotic pathway. Furthermore, we examined the effects of histone to reduce the effective concentration of $InsP_6$.

**Abbreviations:** Δψm, change in mitochondrial membrane potential; IκB, inhibitor of NFκB; NFκB, nuclear factor κB; PARP, poly (ADP-ribose) polymerase; PH domain, pleckstrin homology domain; PI3K, phosphatidylinositol 3-kinase; PtdIns$P_x$ and Ins$P_x$, phosphatidylinositol phosphate and inositol phosphate, respectively (position of phosphates is indicated in the parentheses and '*x*' indicates the number of phosphates); TNF, tumor necrosis factor.

## Materials and methods

*Cell culture and growth assays*

HeLa cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and were treated with $InsP_6$ (Sigma, St Louis, MO) at various concentrations. The number of viable and dead cells was counted on the first, second, third and fourth day after $InsP_6$ treatment. Cells were stained with trypan blue to distinguish the viable and dead cells.

*Soft agar colony formation assay*

This assay was performed using 6 well plates. The bottom layer contained 2 ml of 0.5% agar in medium. The middle layer contained 1 ml of 0.38% agar in medium with $10^3$ HeLa cells. The top layer contained 1 ml of 0.38% agar in medium with various concentrations of $InsP_6$. Cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$.

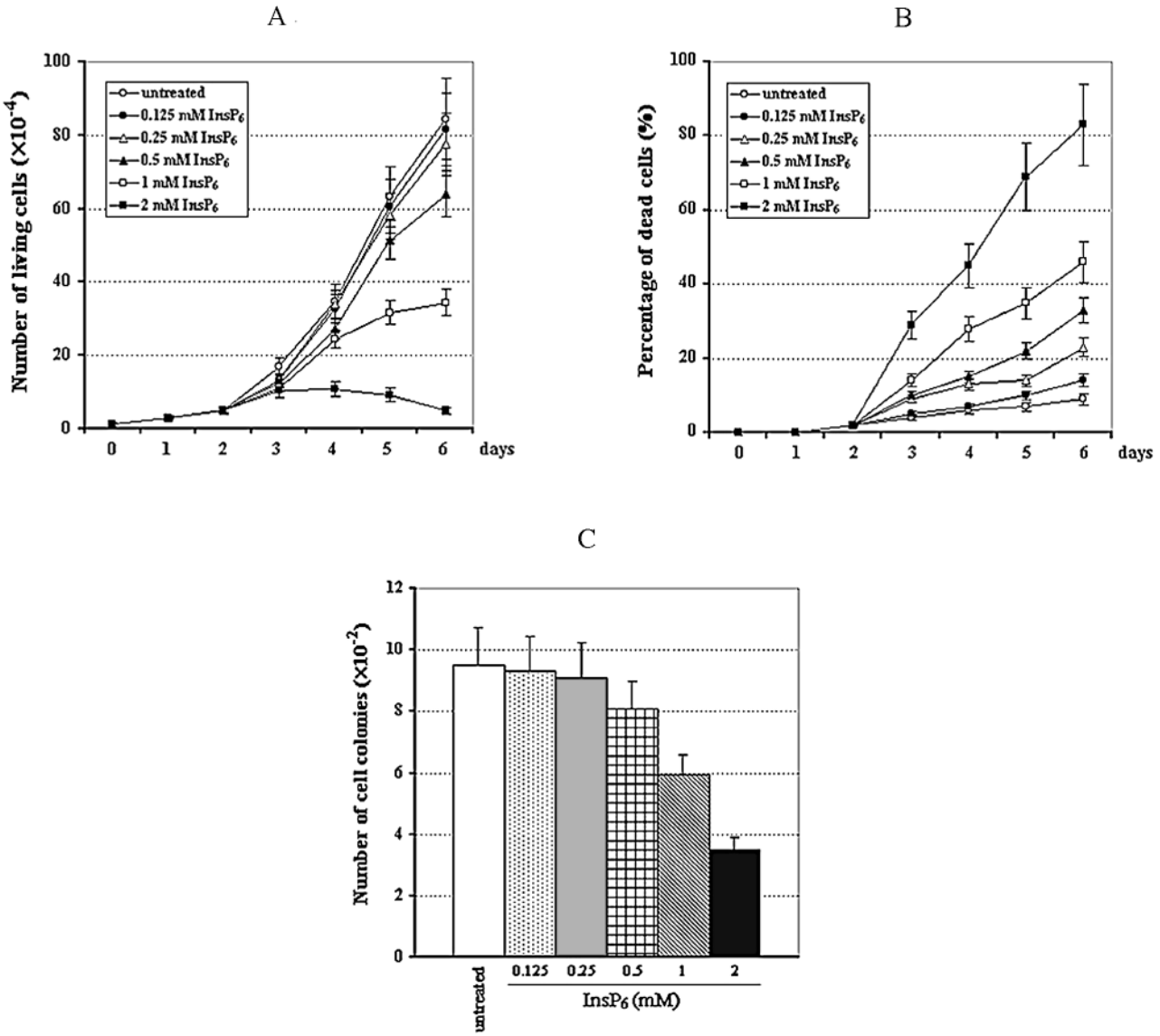



**Fig. 1.** Growth inhibition and apoptosis induction by $InsP_6$. HeLa cells ($2\times10^4$) were starved for 12 h and treated with $InsP_6$ at various concentrations for 2 h. Dulbecco's modified Eagle medium containing 10% fetal bovine serum and $InsP_6$ at various concentrations was then added. Pre-incubation with $InsP_6$ in the absence of serum for 2 h is to facilitate the entry into cells, preventing the complex formation with serum protein. The number of living cells (**A**) and the percentage of dead cells (**B**) were counted on the first, second, third and fourth day after $InsP_6$ treatment. Soft agar colony formation assay was performed as described in the Materials and methods. The number of ⩾15-cell-colonies was counted under a microscope at ×100 magnification (**C**). Each panel shows the mean ± SEM of five independent determinations.

*Hoechst and TUNEL staining*

This experiment was performed according to a method described by Negri *et al.* (17). Briefly, HeLa cells were seeded onto coverslips, stimulated, rinsed in PBS, fixed with ice-cold 10% trichloroacetic acid, and washed in cold 70, 80, 90% and absolute ethanol. DNA apoptotic fragments were end-labeled using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI). After washing, the TUNEL-processed samples were incubated with Hoechst 33258 (Sigma).

*Flow cytometry*

Flow cytometry detection of apoptotic cells was performed as described by Yoshida *et al.* (18). Briefly, pretreated HeLa cells were harvested and suspended in 1 ml of hypotonic fluorochrome solution (50 µg/ml propidium iodide in 0.1% sodium citrate containing 0.1% Triton X-100). Cell suspensions were placed at 4°C in the dark overnight before flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured using an EPICS XL flow cytometer.

*Immunoblotting*

Stimulated cells were incubated with lysis buffer [20 mM HEPES buffer pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 50 mM sodium fluoride, 40 mM sodium β-glycerophosphate, 2 mM sodium orthovanadate, 30 mM sodium pyrophosphate and a cocktail of protease inhibitors (see below)] and scraped. Samples were separated by SDS–PAGE and transferred to a polyvinylidene difluoride sheet. After blocking with 5% skimmed milk in Tris-buffered saline (150 mM NaCl and 20 mM Tris–HCl, pH 7.2), the sheets were incubated with the first antibody. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG antibody (Amersham, Buckinghamshire, UK). The bound antibodies were visualized using the ECL system (Amersham). The cocktail of protease inhibitors contained 10 µM aprotinin, 10 µM pepstatin A, 10 µM leupeptin and 1 mM *p*-amidinophenyl methanesulfonyl fluoride (PMSF).

*Nuclear extract*

After washing with PBS, the cells were incubated in 400 µl buffer A (10 mM HEPES buffer pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and the cocktail of protease inhibitors) on ice for 15 min. Nonidet P-40 at 10% (25 µl) was then added and the cells vortexed for 10 s. After centrifugation for 1 min at 12 000 r.p.m., the resulting pellet was collected, suspended in 50 µl of buffer B (20 mM HEPES buffer pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and the cocktail of protease inhibitors) and incubated on ice for 15 min with occasional vortex

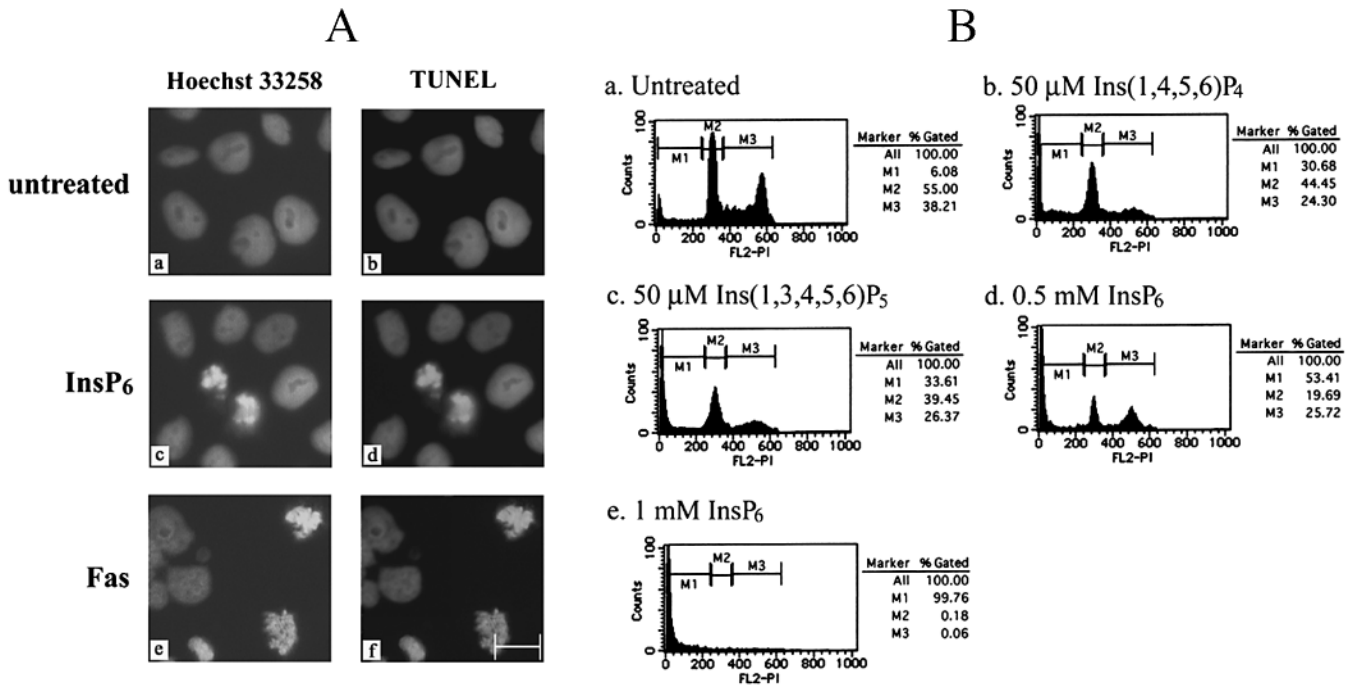



**Fig. 2.** (**A**) Hoechst and TUNEL staining. HeLa cells ($1\times10^6$) were seeded on coverslips, starved for 12 h and treated with 1 mM $InsP_6$ (c and d) or 5 μg/ml Fas (e and f) for 6 h. Hoechst and TUNEL staining were carried out as described in the Materials and methods. The experiment was repeated three times. (**B**) Flow cytometry detection of apoptotic cells. HeLa cells ($1\times10^6$) were starved for 12 h and then stimulated with 50 μM Ins(1,4,5,6)$P_4$ (Cell Signals, Lexington, KY) (b), or 50 μM Ins(1,3,4,5,6)$P_5$ (Cell Signals) (c), 0.5 mM or 1 mM $InsP_6$ (d and e) for 48 h. The procedures were described in the Materials and methods. This experiment was repeated five times.

mixing. After a final centrifugation of the sample for 10 min at 12 000 r.p.m., the supernatant was taken as the nuclear extract.

*Luciferase assay*

HeLa cells were transfected with NFκB-luc (Clontech, Palo Alto, CA). Luciferase assay was performed using the luciferase assay system (Promega). Briefly, after washing with PBS, 400 μl of lysis buffer was added to the cells, followed by a single freeze–thaw to ensure complete lysis. The culture dish was rocked several times, scraped and the contents transferred to a microcentrifuge tube. The tube was vortexed for 15 s and centrifuged at 12 000 *g* for 2 min. The resulting supernatant was collected and added to a luminometer tube containing luciferase assay reagent. The tube was then read for 10 s to measure luciferase activity.

*PI3K assay*

Stimulated HeLa cells were lysed in a buffer (20 mM Tris–HCl buffer pH 8.0, 137 mM NaCl, 1 mM $MgCl_2$, 10% Nonidet P-40, 1 mM dithiothreitol, 0.4 mM sodium orthovanadate and 1 mM PMSF). The cell lysate supernatant was collected by centrifugation and incubated with monoclonal anti-PI3K (p85α) antibody immobilized with protein G–Sepharose. The beads were washed with PBS containing 1% Nonidet P-40 and 1 mM dithiothreitol, with a solution consisting of 0.1 M Tris–HCl buffer (pH 7.6), 0.5 M LiCl and 1 mM dithiothreitol, and finally with a solution containing 10 mM Tris–HCl buffer (pH 7.6), 0.1 M NaCl and 1 mM dithiothreitol. This was followed by incubation with 0.5 mg/ml phosphoinositide fraction containing PtdIns(4,5)$P_2$. Reaction buffer (50 mM $MgCl_2$, 100 mM HEPES buffer pH 7.6, 250 μM ATP containing 5 μCi of [γ-$^{32}$P]ATP) was added and incubated at 30°C for 10 min. The assay was continued as described by Chen *et al.* (12).

*PtdIns(3,4,5)$P_3$ production assay*

[$^{32}$P]Orthophosphate-labeled HeLa cells were pre-treated with buffer A (30 mM HEPES buffer pH 7.4, 110 mM NaCl, 1 mM $MgCl_2$ and 10 mM glucose) containing 25 μM LY294002 or 1 mM $InsP_6$ for 2 h at 37°C, and incubated in buffer A containing 100 ng/ml TNF or insulin for 5 min. This assay was continued as described by Hidaka *et al.* (19).

*Immunofluorescence*

The stimulated HeLa cells were fixed using a solution of 4% paraformaldehyde in PBS. After washing in PBS, the fixed HeLa cells were treated with 100 mM glycine and 0.2% Triton X-100 in PBS. Samples were incubated in 0.1% bovine serum albumin in PBS to block non-specific binding. Polyclonal anti-Akt antibody (New England BioLabs, Beverly, MA) was applied as the first antibody, followed by FITC-conjugated goat F(ab')$_2$ anti-rabbit IgG (Biosource, Camarillo, CA) as the second antibody.

*Δψm analysis*

Δψm analysis was carried out as described by Yoshida *et al.* (20). Briefly, the cell pellet was suspended in 250 μl of 20 nM DiOC6(3) in PBS and incubated in an incubator for 15 min. Δψm was measured using an EPICS XL flow cytometer.

*Mitochondrial fraction*

This assay was carried out according to the MBL cytochrome c kit protocol. Briefly, cells were homogenized in 200 μl of ice-cold solution containing 10 mM Tris–HCl (pH 7.5), 0.3 M sucrose, and the cocktail of protease inhibitors, followed by centrifugation at 100 000 *g* at 4°C for 60 min. The supernatant was collected as the cytosol fraction. The precipitate was resuspended in 200 μl ice-cold solution containing 10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl and protease inhibitor mixture, sonicated with an ultra sonicator, and centrifuged at 10 000 *g*, 4°C for 30 min. The supernatant was collected as the mitochondrial fraction.

*$InsP_6$ internalization*

Pretreated cells were labeled with 0.5 μCi of [$^3$H]$InsP_6$ for 6 h. After washing with PBS, they were incubated with 0.8 ml of 2 M perchloric acid containing 1 mg/ml $InsP_6$ for 15 min on ice. Then 0.4 ml of 1 M potassium carbonate and 5 mM EDTA was added and a freeze–thaw cycle was carried out twice. The mixture was kept at 4°C for 2 h, then centrifuged at 15 000 r.p.m. for 15 min. The resulting supernatant was applied to a strong anion exchange column (Whatman, Kent, UK), mounted on a high performance liquid chromatography system at a flow rate of 1 ml/min. Fractions were collected at 1.1 min intervals, followed by liquid scintillation counting after mixing with 4 ml of ultima-flo (Packard, Grönigen, The Netherlands).

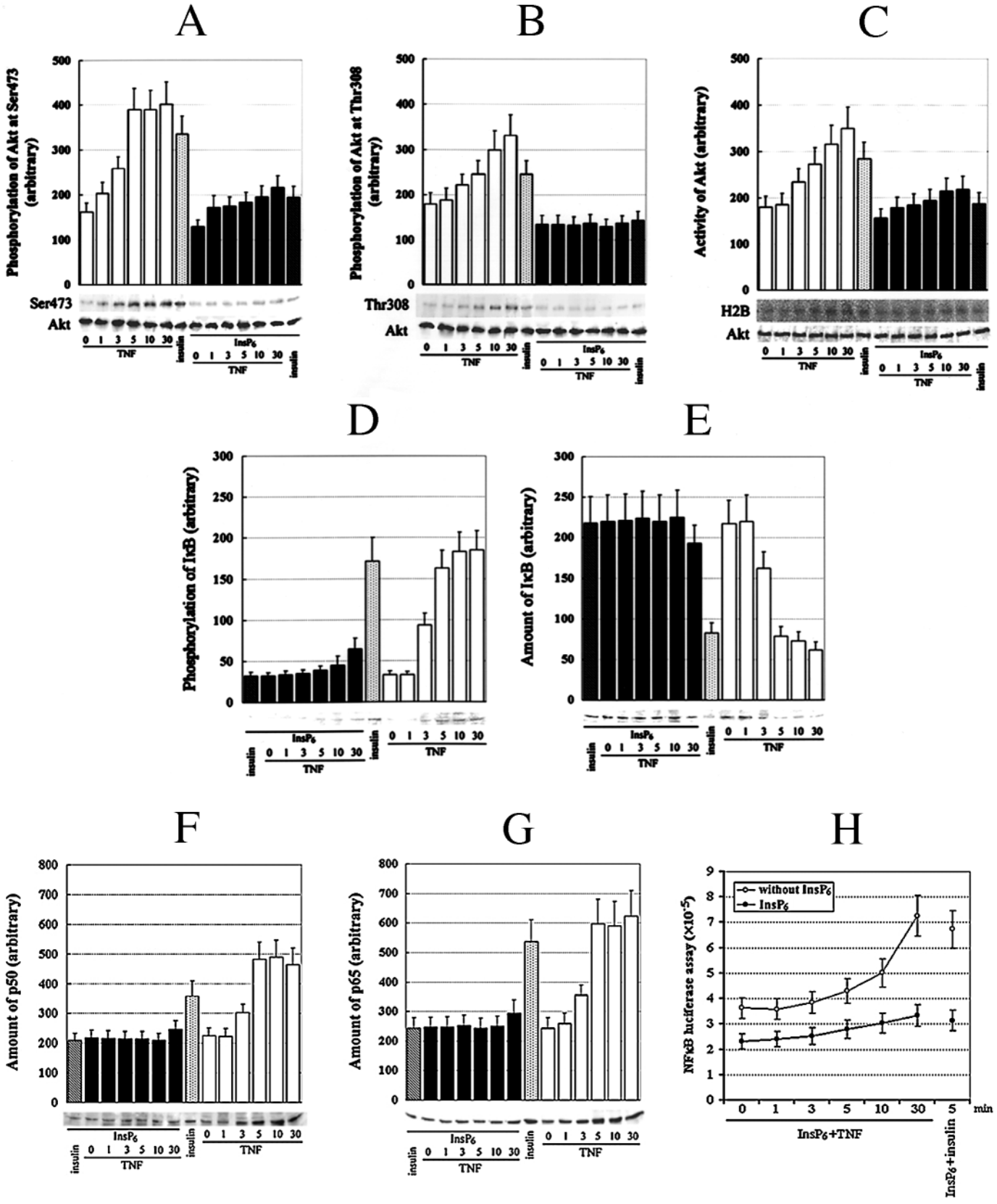
## Results

### *$InsP_6$ inhibited the growth and induced apoptosis of HeLa cells*

HeLa cells were cultured at an initial density of $2\times10^4$ cells in each well of a 6 well culture dish for 2 days. Various concentrations of $InsP_6$ were added to the well, followed by further cultivation for up to 4 days (6 days after the initiation of culture). The growth of HeLa cells was inhibited by $InsP_6$,

and cell death was induced as well (Figure 1A and B). The increase in dead cells was evident at concentrations as minimal as 0.25 mM $InsP_6$, and increased concentrations increased the effect concomitantly. The inhibition of the proliferation of HeLa cells by $InsP_6$ was also seen in an anchorage-free colony formation assay in soft agar (Figure 1C). Such an effect was not observed with inositol hexakissulfate (data not shown), indicating the specific effect of $InsP_6$.

Cell death caused by $InsP_6$ appeared to be apoptosis, since Hoechst 33258 staining of cells treated with $InsP_6$ showed



nuclear fragmentation in a manner similar to cells treated with Fas, which was used as a positive control. Also, the cells stained with TUNEL (Figure 2A). Furthermore, flow cytometric analysis of cells (both dish-attached and floating cells) stained with propidium iodide confirmed the presence of nuclear fragmentation in cells treated with $InsP_6$, as shown in Figure 2B. This also showed that lower concentrations of $InsP_4$ and $InsP_5$ were effective at causing apoptosis. Chelation of divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ by millimolar concentrations of $InsP_6$ was not a cause of apoptosis, because supplementing with $Ca^{2+}$ and $Mg^{2+}$ did not change the results (data not shown). Furthermore, addition of EDTA at millimolar concentrations for up to 4 days to chelate the cations did not induce cell death (data not shown).

*$InsP_6$ inhibited cell survival signaling through the Akt–NFκB pathway*

Apoptosis can be the result of either or both of inhibition of the cell survival signal and the direct induction of apoptosis. Thus, we first examined whether $InsP_6$ inhibits cell survival signaling. For this purpose, we examined whether $InsP_6$ inhibits the TNF–PI3K–Akt–NFκB pathway, which has been shown to stimulate cell survival in SAS cells by us (14) and in 293 and HeLa cells by Ozes *et al.* (13).

Phosphorylation of Akt at both Ser473 and Thr308 was seen within 1 min of stimulation with TNF at 100 ng/ml, and reached maximal levels within 10 min. Stimulation with insulin at 100 ng/ml for 5 min provided similar results (Figure 3A and B). Pre-incubation with 1 mM $InsP_6$ prevented the Akt phosphorylation at both sites (Figure 3A and B). *In vitro* Akt activity was assayed using the phosphorylation of histone 2B (H2B) as the substrate. The results were apparently parallel to those of the phosphorylation of Akt; almost maximal Akt activity was seen at 10 min, and pre-incubation with 1 mM $InsP_6$ abolished the Akt activity (Figure 3C).

It has been reported that Akt activated by phosphorylation can then induce the phosphorylation of IKKs, mainly IKKβ, which later induces the phosphorylation and degradation of IκB (21). Considering that Akt is upstream of this pathway, we needed to check whether this pathway was also inhibited. Stimulation with 100 ng/ml TNF showed IκB phosphorylation in 3 min, which reached maximal levels in 10 min, whilst IκB degradation was seen in 3 min (Figure 3D and E). Similar results were seen in cells stimulated with 100 ng/ml insulin. IκB phosphorylation and degradation by 100 ng/ml TNF or insulin was abolished by pre-incubation with 1 mM $InsP_6$ (Figure 3D and E).

Following IκB phosphorylation and degradation, NFκB is free to translocate to the nucleus (21,22). We also noticed this phenomenon in the present results, in that the amount of NFκB (p65 and p50) was increased in the nucleus after 100 ng/ml TNF stimulation. This translocation was totally inhibited in the cells pre-incubated with 1 mM $InsP_6$ (Figure 3F and G). Results from the NFκB-luciferase transfected HeLa cells also agreed with this result (Figure 3H). Treatment with TNF increased the NFκB transcriptional activity gradually, but the 1 mM $InsP_6$-pre-incubated sample showed only a slight rise in NFκB activity. $InsP_6$ also had the same capacity of inhibiting insulin-induced NFκB nuclear translocation and activity (Figure 3H).

Cell survival is also promoted by activation of the mitogen-activated protein kinase (MAPK) pathway (23,24). $InsP_6$ had little effect on this pathway as assessed by the phosphorylation of MAPK (data not shown), indicating that the inhibition by $InsP_6$ is specific to the Akt cell survival pathway.

*$InsP_6$ did not affect the activity of PI3K and PtdIns(3,4,5)$P_3$ production*

An investigation as to whether $InsP_6$ affects the activity of PI3K, responsible for producing PtdIns(3,4,5)$P_3$, was performed. Tyrosine phosphorylation of p85, a regulatory subunit of type 1 PI3K, was increased by stimulation with 100 ng/ml TNF or insulin. Pre-incubation with 25 μM LY294002, a PI3K inhibitor, inhibited the phosphorylation of p85, but pre-incubation with $InsP_6$ was ineffective (Figure 4A). The ability of PI3K to convert PtdIns(4,5)$P_2$ to PtdIns(3,4,5)$P_3$ was enhanced by immunoprecipitates of the anti-p85 subunit prepared from cells stimulated with TNF or insulin. Pre-incubation with 25 μM LY294002 diminished this effect, but pre-incubation with 1 mM $InsP_6$ did not cause any reduction in the activity (Figure 4B). [$^{32}$P]Orthophosphate-labeled HeLa cells also showed the same result; PtdIns(3,4,5)$P_3$ production was upregulated by stimulation with 100 ng/ml of TNF or insulin. Pre-incubation with 25 μM LY294002 decreased this production but pre-incubation with 1 mM $InsP_6$ had no effect (Figure 4C).

*$InsP_6$ caused the inhibition of Akt membrane translocation*

When PtdIns(3,4,5)$P_3$ is produced in the cell membrane, Akt, present mainly in the cytosol, moves to the cell membrane by binding to PtdIns(3,4,5)$P_3$ via its PH domain. This translocation was seen after stimulation with 100 ng/ml TNF or insulin, and was prevented by pre-incubation with 25 μM LY294002, a potent PI3K inhibitor. Interestingly, the same results were obtained in HeLa cells pre-incubated with 1 mM $InsP_6$ (Figure 5), in spite of little inhibition of PtdIns(3,4,5)$P_3$ production.

*Direct effects of $InsP_6$ on events relating to cell apoptosis*

We then examined whether $InsP_6$ directly activates events relating to apoptosis. We first analyzed Δψm in cells treated with $InsP_6$. We found that 0.5 mM $InsP_6$ lowered Δψm by ~50%. A more dramatic change was seen when 1 mM $InsP_6$ was used (Figure 6A). Cytochrome c release was observed after 6 h treatment with 0.5 mM $InsP_6$ and 12 h treatment caused more cytochrome c release. More cytochrome c release

**Fig. 3.** Phosphorylation and activity of Akt. HeLa cells ($1\times10^6$) were starved for 12 h and treated with or without 1 mM $InsP_6$ for 2 h, followed by 100 ng/ml TNF stimulation for 1, 3, 5, 10, 30 min or 100 ng/ml insulin for 5 min. Cell lysates were immunoprecipitated by polyclonal anti-Akt antibody (New England BioLabs), coupled to protein G–Sepharose (Amersham) for detecting Akt phosphorylation (**A** and **B**). The precipitates were analyzed by immunoblotting using polyclonal anti-phospho-Akt (Ser473) (New England BioLabs) (A) or polyclonal anti-phospho-Akt (Thr308) (New England BioLabs) (B). (**C**) The immunoprecipitates were assayed for kinase activity using H2B and [γ-$^{32}$P]ATP as substrates (14). The membranes were reprobed for Akt using polyclonal anti-Akt (New England BioLabs). To detect the phosphorylation and degradation of IκB, cell lysates were analyzed by immunoblotting using polyclonal anti-phospho-specific-IκB (New England BioLabs) (**D**) or polyclonal anti-IκB (Santa Cruz Biotechnology, Santa Cruz, CA) (**E**). To detect NFκB nuclear translocation, nuclear extracts, prepared as described in the Materials and methods, were analyzed by immunoblotting using polyclonal anti-NFκB p50 (Santa Cruz Biotechnology) (**F**) or polyclonal anti-NFκB p65 (Santa Cruz Biotechnology) (**G**). To detect NFκB nuclear translocation and NFκB-luciferase activity, luciferase assay was performed as described in the Materials and methods (**H**). Each panel shows the typical results and the mean ± SEM of five independent determinations.

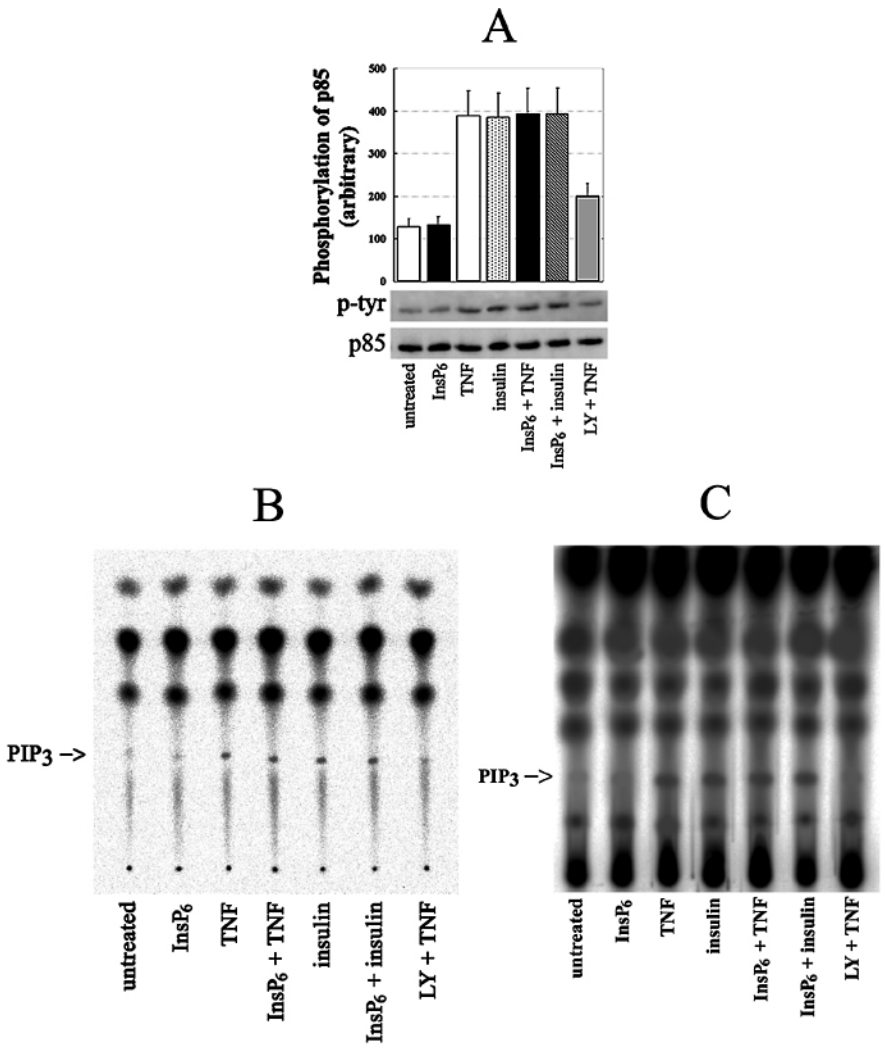



**Fig. 4.** Phosphorylation and activity of PI3K and PtdIns(3,4,5)$P_3$ production. HeLa cells ($1\times10^6$) were starved for 12 h and pretreated with or without 1 mM $InsP_6$ or 25 μM LY294002 for 2 h, followed by 100 ng/ml TNF or 100 ng/ml insulin for 5 min. (**A**) Cell extracts were immunoprecipitated by monoclonal anti-PI3K (p85α) (PharMingen, San Diego, CA), coupled to protein G–Sepharose (Amersham). The precipitates were analyzed by immunoblotting using monoclonal anti-phosphotyrosine (Upstate Biotech, NY). Each panel shows the typical results and the mean ± SEM of five independent determinations. (**B** and **C**) PI3K and PtdIns(3,4,5)$P_3$ production assays were performed as described in the Materials and methods. The experiments were repeated three times.

was detected after treatment with higher concentrations of $InsP_6$ (Figure 6B).

To examine whether $InsP_6$ activates the apoptotic machinery, we studied the cleavage of caspase 9 (caspase recruiting domain), caspase 3 (downstream caspase), and poly (ADP ribose) polymerase (death substrate, abbreviated as PARP). Degradation and cleavage of caspase 9, 3 and PARP were detected slightly after treatment with 0.5 mM $InsP_6$ (Figure 7). Much more degradation and cleavage was seen after 6 h treatment with 1 mM $InsP_6$. The degradation and cleavage were increased by treatment for 12 h.

*$InsP_6$ internalization and dephosphorylation*

To determine how extracellularly applied $InsP_6$ affects the events observed above, HeLa cells were cultured with 1 mM $InsP_6$ (containing 0.5 μCi [$^3$H]$InsP_6$) for 6 h. The extract prepared from perchloric acid treated cells was analyzed by applying it to a strong anion exchange column, followed by scintillation counting. As shown in Figure 8, [$^3$H]$InsP_6$ was internalized and dephosphorylated into $InsP_3$, $InsP_4$ and $InsP_5$. However, pre-incubation with colchicine, a pinocytosis inhibitor, did not produce the dephosphorylated forms of $InsP_6$ (Figure 8). [$^3$H]$InsP_6$ was incubated for 6 h with culture medium prepared just in the same way as for the analysis of [$^3$H]inositol phosphates, but with no addition of an isotope $InsP_6$, followed by the analysis of [$^3$H]inositol phosphates. There was little production of lower [$^3$H]inositol phosphates in the presence or absence of colchicines, indicating that they were the products inside cells.

*Effect of histone to lower the effective concentration of $InsP_6$*

Ozaki *et al.* (25) successfully used histone for intracellular delivery of chemically synthesized analogs of phosphoinositides and inositol phosphates. Therefore, we investigated the effect of histone on $InsP_6$-induced apoptosis, as assessed by nuclear staining with propidium iodide followed by a flow cytometry. As shown in Figure 9, $InsP_6$ at concentration of 100 μM exhibited a partial induction of apoptosis, but the inclusion of histone enhanced the $InsP_6$ action to the similar extent as that observed at 1 mM (see also Figure 2B).

## Discussion

There have been many efforts to induce apoptosis in cancer cells, by activating Fas (26), Apaf-1 (20), apoptosis-inducing

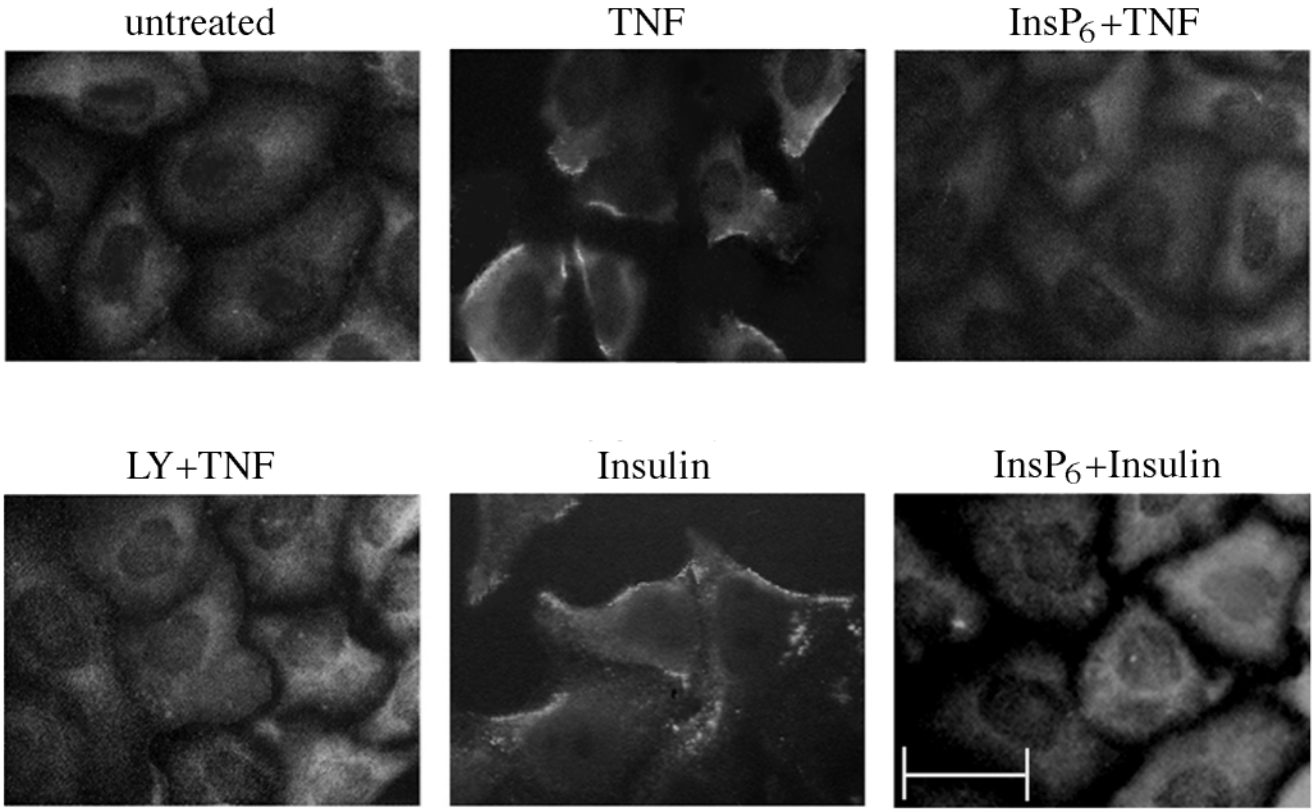



**Fig. 5.** Akt membrane translocation. HeLa cells ($1\times10^6$) were seeded on coverslips, starved for 12 h and pretreated with or without 1 mM $InsP_6$ or 25 μM LY294002 for 2 h, followed by 100 ng/ml TNF or 100 ng/ml insulin for 5 min. Immunofluorescence was carried out as described in the Materials and Methods. This experiment was repeated three times.

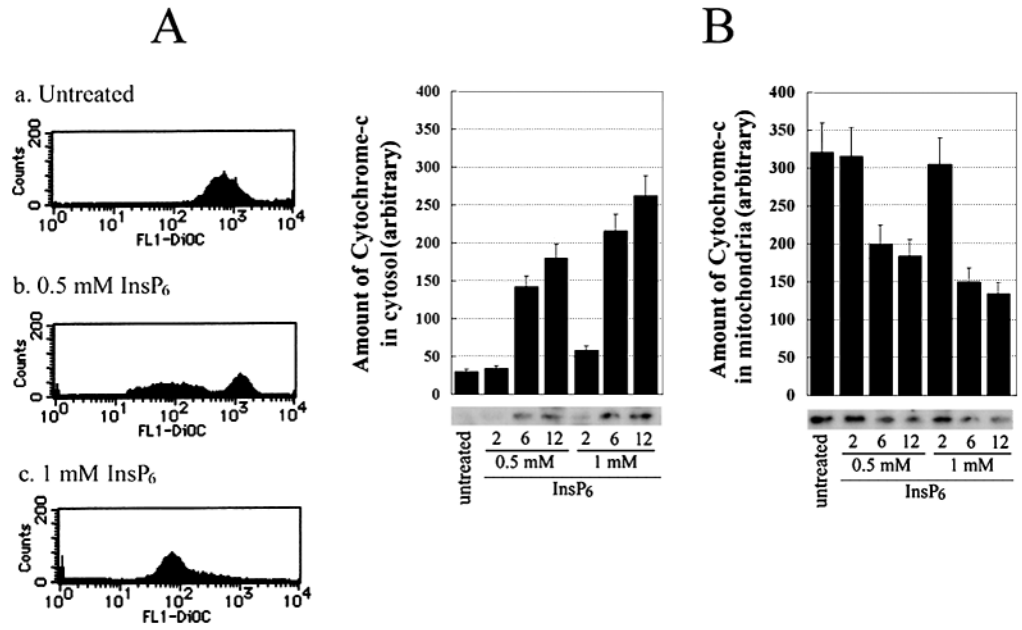



**Fig. 6.** Δψm and cytochrome c release. (**A**) HeLa cells ($1\times10^6$) were starved for 12 h and then stimulated with 0.5 or 1 mM $InsP_6$ for 6 h. Cells were analyzed for Δψm as described in the Materials and methods. The experiment was repeated three times. (**B**) HeLa cells ($5\times10^6$) were starved for 12 h and then stimulated with 0.5 or 1 mM $InsP_6$ for 2, 6 or 12 h. Cells were collected in PBS. Cytosol and mitochondrial fractions were obtained as described in the Materials and methods and analyzed by immunoblotting using monoclonal anti-cytochrome c (PharMingen). Each panel shows the typical results and the mean ± SEM of five independent determinations.

factor (27), and inhibiting the survival pathway via mechanisms independent of (28), or dependent on (14,29), Akt activation. It has been reported that inositol phosphates, which are recognized as second messengers inside cells, have the potential to inhibit cell growth, especially when $InsP_4$, $InsP_5$ and $InsP_6$ are extracellularly applied (16).

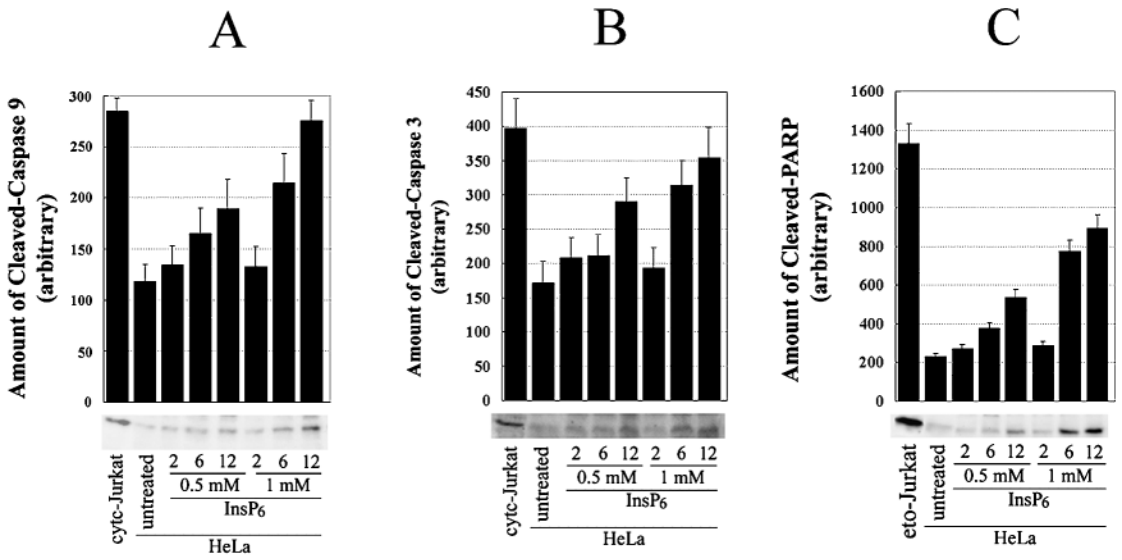



**Fig. 7.** Caspase 9, caspase 3 and PARP. HeLa cells ($1\times10^6$) were starved for 12 h and then stimulated with 0.5 or 1 mM $InsP_6$ for 2, 6 or 12 h. Cell extracts were analyzed by immunoblotting using polyclonal anti-cleaved-caspase-9 (Asp330) (New England BioLabs) (**A**), polyclonal anti-cleaved-caspase-3 (Asp175) (New England BioLabs) (**B**), polyclonal anti-cleaved-PARP (Asp214) (New England BioLabs) (**C**). Cytc-Jurkat and eto-Jurkat were included in the apoptosis sampler kit as positive controls. Each panel shows the typical results and the mean ± SEM of five independent determinations.

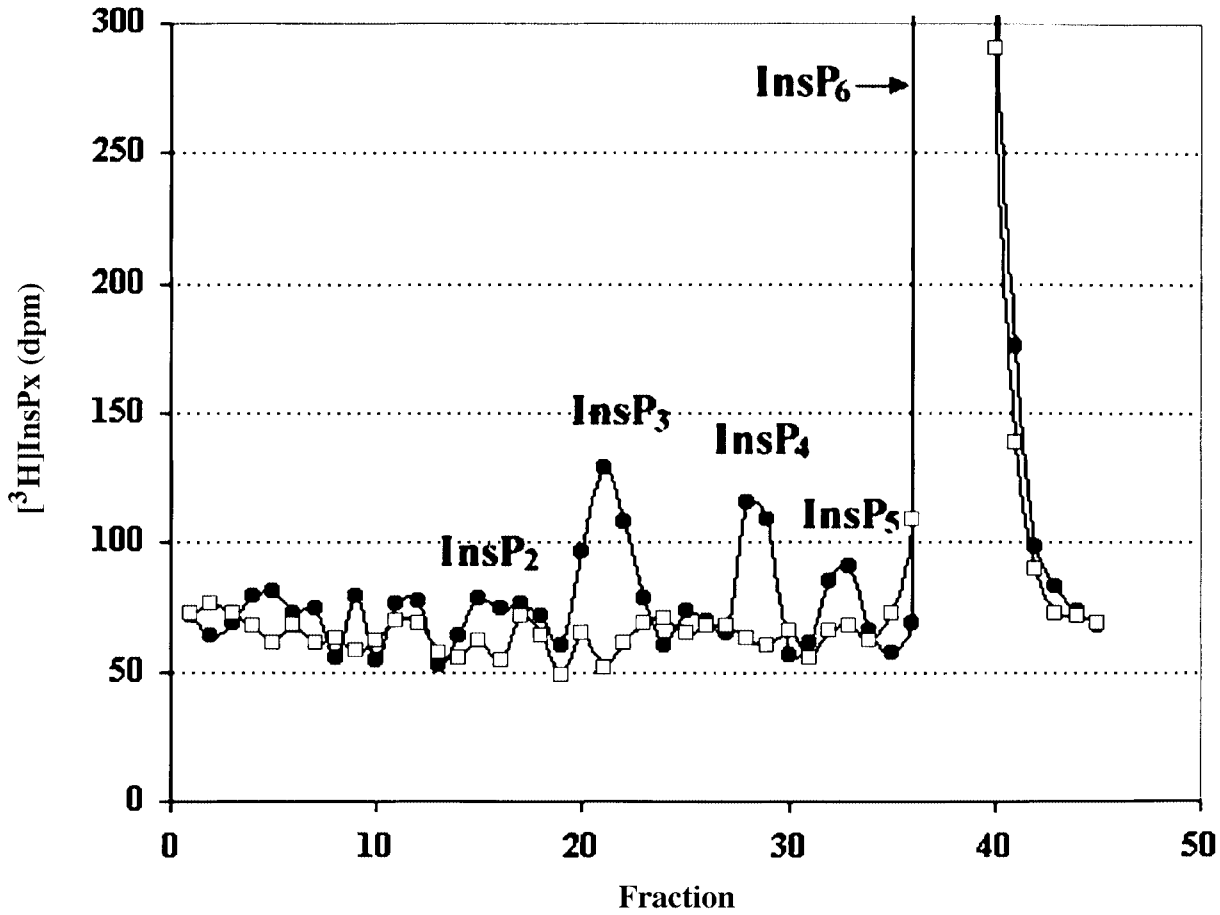



**Fig. 8.** $InsP_6$ internalization and dephosphorylation. HeLa cells ($1\times10^6$) were starved for 12 h and then incubated with or without 10 μg/ml colchicine for 2 h. The cells were then stimulated with 1 mM $InsP_6$ for 2 h, followed by the addition of 0.5 μCi of [$^3$H]$InsP_6$ for 6 h. The procedures used were as described in the Materials and methods. The fractions of InsP*x* indicated in the figure were examined by applying an authentic [$^3$H]InsP*x* to the same column in advance. Most of the radioactivity corresponding to $InsP_6$ appears to be originated from the extracellular space even after extensive washing. Open squares indicate the results obtained from the cells pretreated with colchicines. The figure represents a typical analysis and the experiment was repeated three times.

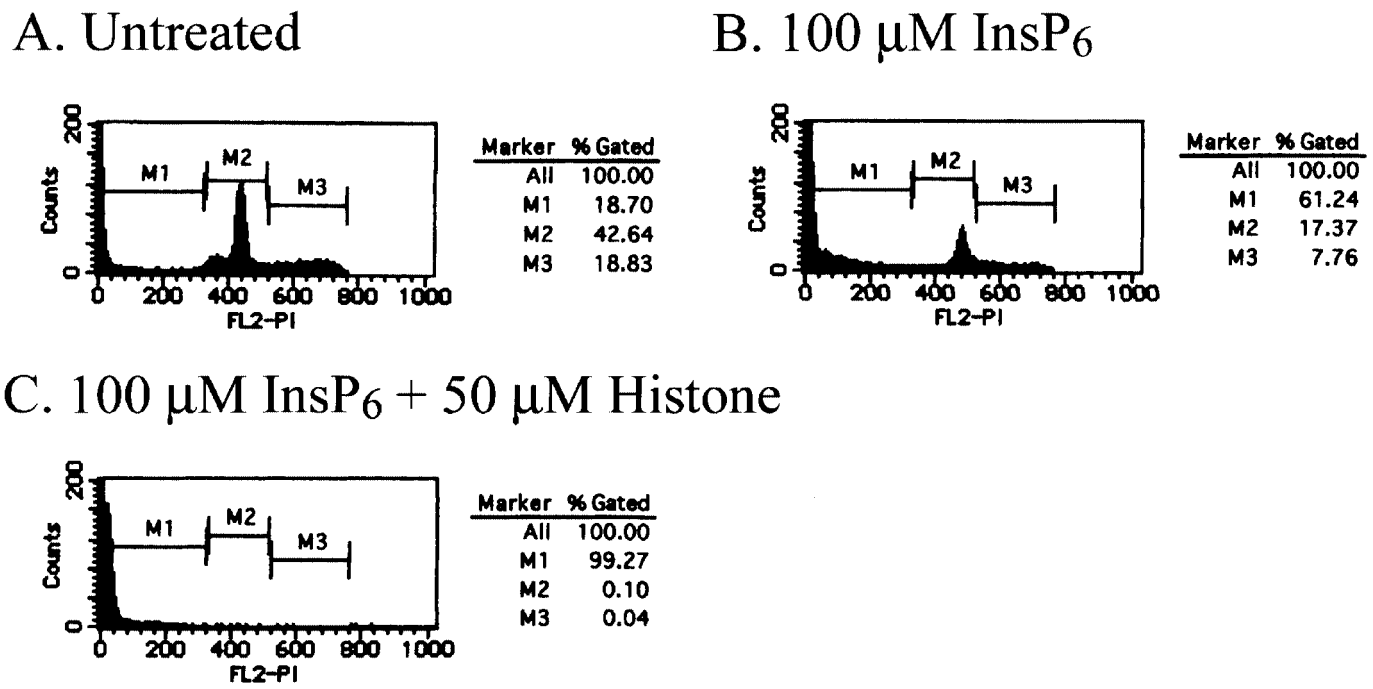



**Fig. 9.** Flow cytometry detection of apoptotic cells. HeLa cells ($1\times10^6$) were starved for 12 h and then stimulated with none (**A**), 100 μM $InsP_6$ (**B**) or 100 μM $InsP_6$ plus 50 μM histone (**C**) for 48 h. Histone alone exhibited the same pattern as that seen in (A). The procedures were described in the Materials and methods. This experiment was repeated five times.

The present results show that, besides having the potential to inhibit cell growth in both anchorage-dependent culture and anchorage-independent soft agar assays, InsPxs also have the potential to induce cell death. The ability of $InsP_6$ to cause cell death has already been described by another group (11) but the mechanisms underlying this are not clearly understood. It has been shown that the Akt pathway is a major pathway that can induce cell survival (13,21). Therefore, we first investigated the effect of $InsP_6$ on the Akt pathway.

Akt phosphorylated at Ser473 and Thr308, an active form, was upregulated by TNF stimulation, whilst pre-incubation with $InsP_6$ inhibited the phosphorylation. This effect of $InsP_6$ was also shown in an *in vitro* model assay using H2B as a substrate. Akt activated by phosphorylation has many downstream effects (30), one of which is the phosphorylation of IκB and consequent release of NFκB. Released NFκB is translocated to the nucleus, which is an important stage in the continuation of this survival pathway (31,32). We also noticed that pre-incubation with $InsP_6$ prior to TNF stimulation inhibited all of the activation signals mentioned above; the phosphorylation and degradation of IκB and NFκB translocation were abolished. We further pursued this investigation on NFκB-luciferase activity, and again we found that after pre-incubation with $InsP_6$, the NFκB-luciferase activity was inhibited.

It was clear that $InsP_6$ could inhibit the Akt–NFκB pathway, but it was necessary to investigate the effect of $InsP_6$ on PI3K. This is a key regulator upstream of Akt (33), involved in a complex series of events, including genetic alterations, leading to aberrant cell cycle, progression and inhibition of apoptosis (34,35). In the present study, we found that $InsP_6$ did not inhibit the phosphorylation of p85, a regulatory subunit of type1 PI3K. We also found that $InsP_6$ did not decrease PI3K activity, as assessed by both an *in vitro* kinase assay using immunoprecipitates of anti-p85 antibody as an enzyme source and PtdIns(3,4,5)$P_3$ production in intact cells labeled with [$^{32}$P]orthophosphate. However, Akt membrane translocation, probably caused by the binding of the Akt PH domain to PtdIns(3,4,5)$P_3$ produced in the plasma membrane, was inhibited by pre-incubation with $InsP_6$. Another group reported the inhibition of this process by Ins(1,4,5,6)$P_4$ (16), probably because of the binding of Ins(1,4,5,6)$P_4$ to Akt so that the PH domain of Akt cannot bind to PtdIns(3,4,5)$P_3$. We reported previously the binding affinity between a variety of InsPxs and the PH domain of Akt (36,37), showing that the PH domain of Akt binds preferably to Ins(1,4,5,6)$P_4$, interfering with the binding of Akt to PtdIns(3,4,5)$P_3$.

Extracellularly applied $InsP_6$ is effective in the events observed in the present study, entering cells pinocytotically and then being further dephosphorylated into the appropriate InsPs. This was proved by analyzing [$^3$H]inositol-labeled InsPxs in extracts of intact cells incubated with [$^3$H]$InsP_6$. [$^3$H]$InsP_3$, [$^3$H]$InsP_4$ and [$^3$H]$InsP_5$ metabolized from [$^3$H]$InsP_6$ were found to be accumulated inside cells, but we have not yet analyzed which isomers are dominant. Morrison *et al.* (38) have suggested that $InsP_6$ kinase 2, which phosphorylates $InsP_6$ to $InsP_5$-PP, might be important in apoptosis. We also found a small radioactive peak, corresponding to $InsP_5$-PP ($InsP_7$) in HeLa cells incubated with [$^3$H]$InsP_6$ (data not shown), indicating that this inositol polyphosphate might also be involved in the apoptosis. These metabolites from [$^3$H]$InsP_6$ would be produced inside cells, because the treatment with colchicine, an inhibitor of pinocytosis, did not produce the metabolites. Furthermore, little metabolites were produced by incubating [$^3$H]$InsP_6$ with the culture medium prepared in the same way as for the assay of [$^3$H]inostol phosphates. Since the radioactivities of [$^3$H]$InsP_6$ were much the same between the cell extracts treated with or without colchicine, the majority of the radioactivity would be due to the extracellular binding.

In response to death stimuli, mitochondrial membranes are

permeabilized (39,40) and cytochrome c is released from the mitochondria (41,42). To find out whether $InsP_6$ is a death stimulator, we investigated the effect of $InsP_6$ on $\Delta\psi m$ without any stimulants, because the presence of stimulants, including serum, always enhances cell survival signals. The results showed that $InsP_6$ itself could cause a decrease in $\Delta\psi m$, indicating that the mitochondria were permeabilized. Although there are reports that Akt is involved in maintaining the integrity of the mitochondrial membrane (43,44), in the present experimental system $InsP_6$ was applied without any stimulation, so that the Akt pathway was not upregulated. The results suggest that $InsP_6$ itself could directly cause the mitochondrial defect. We realize that inhibition of the Akt pathway by $InsP_6$ might play a role in affecting the mitochondria membrane potential as well. We also found increased amounts of cytochrome c in the cytosol and reduced cytochrome c levels in the mitochondria, indicating that cytochrome c was released from the mitochondria. Released cytochrome c induces the cleavage of caspase 9, a caspase recruiting domain, which induces the activation of caspase 3, and consequently the activation of apoptotic substrates (45). Our present results showed that, after treatment with $InsP_6$, caspase 9, caspase 3 and PARP, an apoptotic substrate, were cleaved in a time- and $InsP_6$ concentration-dependent manner. Treatment of HeLa cells with 1 mM $InsP_6$ for 6 h, which was sufficient to cause mitochondrial permeabilization, cytochrome c release, activation of caspase 9, caspase 3 and PARP, was carried out to observe DNA fragmentation by TUNEL.

As mentioned in the introduction, the dephosphorylation of $InsP_6$ to $InsP_{1–5}$ can possibly enhance its anticancer function (16). We tested the potential of the lower forms to induce apoptosis. We treated HeLa cells with Ins(1,4,5,6)$P_4$ or Ins(1,3,4,5,6)$P_5$, and detected apoptosis by flow cytometry. We found that these lower metabolites are more active than $InsP_6$. This result agreed with the report by Razzini *et al.* (16). It suggested that $InsP_6$ enters the cell and is dephosphorylated to lower forms, which are more potent at inducing apoptosis. The lower phosphorylated forms, especially Ins(1,4,5,6)$P_4$, which has the highest affinity for the Akt PH domain (36,37), also inhibit the Akt pathway (16), indicating that lower concentration of these agents could be used for anticancer action. However, $InsP_4$, especially an isomer whose position of the phosphates is defined is expensive. So the trial to reduce the effective concentration of $InsP_6$ was achieved by using histone, the principle to use it is to neutralize the negative charges of the phosphates of $InsP_6$ to facilitate the transport through the plasma membrane (25).

In conclusion, we suggest that $InsP_6$, which is probably pinocytotically transported into cells and subsequently dephosphorylated to $InsP_4$ and $InsP_5$, can inhibit the Akt-NFκB cell survival pathway by inhibiting the translocation of Akt to the plasma membrane and, thus, its activation. It can also induce the apoptotic machinery directly. The next step is to clarify the mechanism by which extracellularly applied $InsP_6$ induces mitochondrial permeabilization.

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