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Title:

A partially degraded product of phytate suppresses the proliferation of HCT116 colorectal cancer cells

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Abstract

Phytate, *myo*-inositol hexaphosphate (IP6), has been shown to possess anti-carcinogenic qualities. IP6 appears to be partially digested in the digestive tract and degraded into inositol di, tri-, and tetra-phosphate (IP2, IP3, and IP4). The present study investigated whether IP3-rich phytate hydrolysate (IP3-RPH), a partially degraded product from phytate play a role in the suppression of cell proliferation using HCT116 colon carcinoma cell line. IP3-RPH suppresses cell proliferation as well as IP6, and the suppression activity of IP3-RPH was higher than that of IP6. Suppression modes clearly differed between IP3-RPH and IP6. Our findings suggest that partially degraded IP6 products are responsible for the suppression of colon carcinogenesis by IP6.

1. Introduction

Phytate, *myo*-inositol-1,2,3,4,5,6-hexaphosphate (IP6), is found in many foods including cereals and legumes (Harland & Oberleas, 1987). Many studies have shown that IP6 suppresses colon carcinogenesis (Fox & Eberl, 2002; Vucenik & Shamsuddin, 2003). However, the mechanisms by which IP6 suppresses colon carcinogenesis are still unclear.

Ingested IP6 is degraded at least partially in the human digestive tract, although IP6 is not hydrolyzed by digestive enzymes in the gastrointestinal tract (Sandberg & Andersson 1988). Phytase in ingested foods (Rapp, Lantzsch, & Drochner, 2001) and/or intestinal bacteria (Wise & Gilburt, 1982) appear to be responsible for IP6 degradation. Researchers have demonstrated that IP6 phosphorous in oatmeal is almost completely removed in the human digestive tract (Cruickshank, Duckworth, Kosterlitz, & Warnock, 1945) and 56% of IP6 is hydrolyzed in the digestive tract of rats (Wise et al., 1982). Thus, epithelial cells in the large intestine are not necessarily exposed to IP6 even if it is ingested.

Our previous study (Suzuki, Nishioka, Ishizuka, & Hara, 2010) investigated how partially degraded IP6 products (prepared using phytase from *Aspergillus ficcum*) affect intracellular signaling in some types of colon cancer cell lines. Some of the resident bacteria in luminal content might produce these degraded products after IP6 ingestion. We found that the partial hydrolysate of IP6 mainly contains inositol tri-phosphate (IP3) with three phosphates on positions 1,2,6 and 2,5,6. As a result, the IP6 hydrolysates activate protein kinase Cβ (PKC) in undifferentiated types of colon

cancer cell lines, probably via recognition by a G-protein coupled receptor (GPCR). Interestingly, intact IP6 or inositol had no effect on the signaling pathway under identical experimental conditions.

Partially degraded IP6 products produced in the gastrointestinal tract might be responsible for the suppression of colon carcinogenesis by IP6. This study investigated whether IP6 and its degraded products had differing effects on cell proliferation, cell-cycle distribution, and cyclin expressions of HCT116 colon cancer cells.

2. Materials and Methods

2.1. Preparation of myo-inositol triphosphate rich phytate hydrolysate (IP3-RPH)

Preparation of IP3-RPH samples was conducted according to a process that we have reported previously (Suzuki et al., 2010). Briefly, IP6 digested by phytase from *Aspergillus ficcum* was desalted using a packed column with a strongly acidic cation-exchange resin, Daiaion PK216. The eluate was concentrated using a rotary evaporator. This partial hydrolysate of phytate was composed mainly of inositol tri-phosphate (IP3) as a mixture of *myo*-inositol with three phosphates on positions 1,2,6 and 2,5,6, as well as minor components of inositol tetra-phosphate (IP4) with four phosphates on positions 1,2,X,6 (X = 3, 4, or 5) and inositol 1,2-diphosphate (IP2). The ratio of IP2, IP3, and IP4 in IP3-RPH is 1 : 8.6 : 3.6 according to the HPLC analysis we have reported previously (Suzuki et al., 2010).

2.2. Cell culture

The HCT116 cell line that had been isolated from a male patient with colon carcinoma (Brattain, Fine, Khaled, Thompson, & Brattain, 1981) was obtained from Dainippon Seiyaku (Cat. No. 08-247, Osaka, Japan). HCT116 cells possess wild-type *p53* gene (Take, Kumano, Teraoka, Nishimura, & Okuyama, 1996) involved in the induction of apoptosis (Lowe, Schmitt, Smith, Osborne, & Jacks, 1993; Shaw, Bovey, Tardy, Sahli, Sordat, & Costa, 1992) and G1 arrest (Kastan, Onyekwere, Sidransky, Vogelstein, & Craig, 1991; Lane, 1992). No mycoplasma was detected in the cultures. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, 12100-046, Gibco®, Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 35 μg/mL penicillin G potassium, and 50 μg/mL streptomycin sulfate and were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

The IEC-6 cell line was obtained (ATCC CRL 1592) from American Type Culture Collection (Manassas, VA, USA) at passage 11. The cell line is derived from normal rat small intestine (Quaroni, Wands, Trelstad, & Isselbacher, 1979). Mycoplasma was not detected in the cultures. The cells were maintained in DMEM supplemented with 5% heat-inactivated FBS, 0.1 U/mL insulin, 35 μg/mL penicillin G potassium, and 100 μg/mL streptomycin sulfate and were incubated at 37°C in a humidified atmosphere 95% air and 5% CO_2.

2.3. Cell proliferation

Cells were seeded at 5×10^2 cells/well on 96-well plates and incubated in DMEM for 24 h. Then, the cells were exposed to DMEM containing either IP6 or

IP3-RPH for 48 h. The number of cells was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's guidelines. The optical density of the culture supernatant was determined at 450 nm for measurement and at 655 nm for reference.

2.4. mRNA expression of cyclins

Cells seeded at 1.7×10^5 cells/well (six-well plate) were cultured for 48 h. Next, the cells were exposed to IP6 or IP3-RPH (0.75 mM in DMEM) for another 48 h. Total RNA was isolated from the cells exposed to IP6 or IP3-RPH using Trizol (Invitrogen, Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from the total RNA using Reverscript I (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with a random 6-mer primer. The cDNA was subjected to PCR using GoTaq master mix (Promega Co., Madison, WI, USA) and the following primer sets: GAPDH (NM_002046.3) forward: 5'-acagtcagccgcatcttctt-3', reverse: 5'-gacaagcttcccgttctcag-3' (annealing temp, 58°C; product size, 259 bp), cyclin D1 (NM_053056.2) forward: 5'-tggtgaacaagctcaagtgg-3', reverse: 5'-tgaggcggtagtaggacagg-3' (annealing temp, 58°C; product size, 258 bp), cyclin D3 (NM_0017603) forward: 5'-atgctggcttactggatgct-3', reverse: 5'-acagagggccaaaaaggtc-3' (annealing temp, 58°C; product size, 393 bp), cyclin E2 (NM_057749.1) forward: 5'-gctggggatgttcaaaaga-3', reverse: 5'-tcttcactgcaagcaccatc-3' (annealing temp, 58°C; product size, 346 bp), and cyclin A2 (NM_001237.3) forward: 5'-aacttcagcttgtgggcact-3', reverse: 5'-aaaggcagctccagcaataa-3' (annealing temp, 58°C;

product size, 326 bp). The PCR reaction was performed for 30 cycles. Each PCR product was separated with 1.5% agarose gel and stained with ethidium bromide. Expression of the product was determined using a UV transilluminator. Data were expressed as the relative band density of each cyclin against GAPDH.

2.5. Cell cycle and 5-bromo-2'-deoxyuridine (BrdU) incorporation

Cells seeded at 1×10^6 cells/dish (10 cm) were cultured for 48 h. Next, the cells were exposed to IP6 or IP3-RPH (0.3, 0.75, or 1.5 mM in DMEM) for another 47 h, after which BrdU (10 μg/mL, MP Biomedicals, LLC.) was added to the culture and incubated for 1 h. For cell cycle analysis, cells were harvested, and cell nuclei were also stained with propidium iodide (PI). The proportions of cells in G0/G1, S, G2/M, and sub G1 phases were determined using a FACSCalibur (Becton, Dickson and Company, Franklin Lakes, NJ, USA). For BrdU incorporation, harvested cells fixed with ethanol were treated with 4 N HCl at 4˚C for 25 min. The cells were washed with PBS after a brief exposure to 50 mM $B(OH)_3$ and 14.7 mM sodium tetraborate. Next, the cells were incubated with anti-BrdU monoclonal antibody (NA20, Calbiochem, EMD Chemicals, Inc., Darmstadt, Germany). After washing, the cells were stained with FITC-conjugated anti-mouse IgG+A+M (H+L; Zymed Laboratories, San Francisco, CA, USA). The cell nuclei were also stained with PI. The proportion of BrdU-incorporating cells to whole cells (PI positive cells) was determined using FACSCalibur.

2.6. Statistics

All results are expressed as means ± SEMs. Comparisons among multiple

groups were analyzed using Tukey–Kramer's test. Comparison with the control was determined using Student's *t*-test. A probability of less than 0.05 was considered significant. All statistical analyses were performed using JMP 5.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Proliferation of HCT116 cells in response to IP6 or IP3-RPH

We investigated whether IP6 and IP3-RPH, partially degraded products of IP6, influenced HCT116 cell proliferation (Fig. 1). Proliferation of HCT116 cells was gradually retarded with increased IP6 concentration (Fig. 1a). Exposure to IP3-RPH also reduced HCT116 cell proliferation (Fig. 1b). Low-dose exposure to IP3-RPH (0.3 mM) was sufficient to decrease cell proliferation, and the suppressive effects were greater than those induced by IP6 doses of 0.3 and 0.5 mM. These results indicate that both IP6 and IP3-RPH suppress HCT116 cell proliferation and that at low doses, IP3-RPH has a stronger suppressing ability than does IP6.

3.2. Cell-cycle distribution of HCT116 cells exposed to IP6

We analyzed cell-cycle distribution in HCT116 cells after they were cultured with IP6. The proportion of cells in the G0/G1 phase was significantly greater among cells exposed to 0.3 mM of IP6 than among cells without exposure to IP6 (Fig. 2a). When cells were cultured with 1.5 mM of IP6, the proportion of cells in the G0/G1 phase was significantly lower among cells exposed to IP6 than among control cells (Fig.

2c). The proportion of cells in the S phase was significantly reduced among cells exposed to 0.3 and 0.75 mM of IP6 (Fig. 2a and 2b). We also identified differences in the proportions of the cells in the M phase among cells exposed to 0.75 mM of IP6 (Fig. 2b). We observed large increases in the proportion of cells in sub G1 among cells exposed to 0.75 and 1.5 mM of IP6.

3.3. Cell-cycle distribution of HCT116 cells exposed to IP3-RPH

The effects of IP3-RPH on cell-cycle distribution among HCT116 cells differed greatly from the effects of IP6 (Fig. 3). IP3-RPH exposure (0.3 and 0.75 mM) significantly increased the proportion of cells in S and sub G1 phases (Fig. 3a and 3b). When cells were exposed to 1.5 mM of IP3-RPH, the proportion of cells in the G0/G1 phase was reduced, and the proportion of cells in G2/M and sub G1 phases was increased (Fig. 3c).

3.4. Expression of cyclin mRNAs in HCT116 in response to 0.75 mM of IP6 or IP3-RPH

Next, we investigated whether expression of cell-cycle-related genes differed by treatment with IP6 and IP3-RPH. Previous studies have reported expression of cyclin D1 (Sutter, Doi, Carnevale, Arber, Weinstein, 1997), cyclin D3 (Bockstaele, Bisteau, Paternot, & Roger, 2009), cyclin E (Sutter et al., 1997), and cyclin A2 (Lee, Kim, Barbier, Fotedar, & Fotedar, 2009) in HCT116 cells. We measured mRNA expression of cyclin D1, cyclin D3, cyclin E2, and cyclin A2 in HCT116 cells exposed to 0.75 mM of IP6 or IP3-RPH (Fig. 4). As expected, we observed significant reduction of cyclin D3

and E2 expressions in cells exposed to IP6 (Fig. 4b and 4c). IP3-RPH significantly reduced cyclin D3 expression, but the rate of reduction was weaker in IP3-RPH-treated cells than in IP6-treated cells (Fig. 4b). No significant reductions in expression of other cyclins occurred in cells treated with IP3-RPH. These results indicate that cell-cycle arrest was relatively weaker in cells exposed to IP3-RPH than in cells exposed to IP6.

3.5. BrdU incorporation in HCT116 cells in response to IP3-RPH

We investigated DNA synthesis in cells exposed to IP3-RPH (Fig. 5). IP3-RPH treatment decreased BrdU incorporation in HCT116 cells. However, we observed no significant reduction in BrdU incorporation in cells treated with 0.3 mM of IP3-RPH, even though IP3-RPH at this concentration clearly suppressed cell proliferation, as shown in Fig. 1.

3.6. *Proliferation and cell-cycle distribution of IEC6 cells in response to IP6 or IP3-RPH*

We also investigated influence of IP6 and IP3-RPH on IEC6 cells derived from normal small intestinal epithelial cells in rats (Quaroni et al., 1979). In cell proliferation assay, the response of IEC6 cells to IP6 or IP3-RPH (Fig. 6a) was similar to those in HCT116 cells (Fig. 1). However, in cell-cycle analysis, almost no cells were found in sub-G1 phase both in IP6- and IP3-RPH-treated cells. Additionally, there was no apparent difference in cell-cycle distribution in IP6- and IP3-RPH-treated cells from that in control (Fig. 6b-d).

4. Discussion

Our results indicate that both IP6 and IP3-RPH suppress proliferation of HCT116 colon carcinoma cells; IP3-RPH yielded much better results at the lowest dose of 0.3 mM. In addition, we found that IP6 promoted cell-cycle arrest in HCT116 cells and reduced cyclin D3 and E2 expression. In contrast, IP3-RPH induced cell death of HCT116 cells. Cell cycle arrest by IP3-RPH was very weak in the cell cycle distribution assay; this result is consistent with results for BrdU incorporation, which yielded no changes in DNA synthesis at low doses of IP3-RPH. Thus, the mechanisms for suppressing proliferation differed between IP6 and IP3-RPH.

Previous studies using animal experiments have reported that IP6 suppresses colorectal carcinogenesis (Fox et al., 2002; Vucenik et al., 2003), but were unable to clarify the precise mechanisms for the results. In contrast, cell culture studies have demonstrated that IP6 induces cell death in many types of cell lines via G0/G1 arrest and S-phase inhibition (El-Sherbiny, Cox, Ismail, Shamsuddin, & Vucenik, 2001), which supports our findings. Inhibition of the NFkappaB pathway is a signal responsible for IP6-induced cell death in HeLa cells (Ferry, Matsuda, Yoshida, & Hirata, 2002). In HCT116 cells, curcumin induces c-jun N-terminal kinase-dependent apoptosis by inhibiting the NFkappaB pathway (Collet & Campbell, 2004). In this study, we found that IP3-RPH also increased the proportion of sub G1 in HCT116 cells. The NFkappaB pathway might also be involved in IP3-RPH-induced HCT116 cell death.

We also investigated influence of IP6 or IP3-RPH on IEC6 cells, derived from normal intestinal epithelial cells. Both IP6 and IP3-RPH suppressed proliferation of IEC6 cells as well as HCT116 cells. However, almost no cell in IEC6 cells was detected in sub G1 phase in cell-cycle distribution analysis. These results indicate that both IP6 and IP3-RPH retard cell cycle progression in intestinal epithelial cells. Cell-cycle arrest by these molecules might be different event from retardation of cell cycle progression. The response seems different between normal and tumor cells. To understand precise mechanisms, it is required to clarify how to recognize IP6 and/or IP3-RPH and subsequent signaling pathways in intestinal epithelial cells.

Our previous study demonstrated how IP6 and IP3-RPH induced different signaling pathways by using specific inhibitors for some signaling molecules (Suzuki et al., 2010). IP3-RPH activates PKC, probably via GPCR, but IP6 does not activate this pathway. IP3 is more likely to induce Ca^{2+} signaling than IP2 or IP4 (Suzuki et al., 2010). Composition analysis revealed that the major component in IP3-RPH is IP3. IP3 in IP3-RPH is considered to be responsible for the suppression of HCT116 cell proliferation. Thus, the differing influences on HCT116 cells from IP6 and IP3-RPH might be a result of recognition and/or intracellular signaling pathways.

It is reported that phytate content is ranging from 0.06 to 2.2% in cereals (Schlemmer, Frolich, Prieto, & Grass, 2009). In this study, we used 1.5 mM of IP6 in the culture studies. The concentration of 1.5 mM in IP6 is equivalent to 0.1% in weight basis. This value is less than phytate contents in many cereals. We suppose that concentration of IP3-RPH derived from IP6 is much less than that of IP6 although

255 precise concentration of IP3-RPH has not been determined so far. It is reported that
256 maximum concentration of IP6 after ingestion of 1.4 g of sodium phytate is around 0.18
257 μM at 4 h post ingestion in humans (Grases, et al. 2001). The influence of IP6 or
258 IP3-RPH at mM level might be limited to within intestinal contents, not after
259 absorption.

260 In the large intestine, intestinal bacteria might produce a substantial amount of
261 IP6 hydrolysates such as IP3-RPH (Wise et al., 1982). The production of IP6
262 hydrolysates might be modulated by the ingestion of probiotic bacteria and/or prebiotics
263 such as dietary fiber and oligosaccharides. To date, no studies have investigated all the
264 types of IP6 hydrolysates produced in the large intestine. It would be very interesting to
265 clarify how food factors modulate the production of IP6 hydrolysates. The IP6
266 hydrolysates used in the present study were produced using phytase from *Aspergillus*
267 *ficcum* (Suzuki et al., 2010). It is likely that many types of fermented foods processed
268 worldwide using this kind of mold contain large quantities of IP6 hydrolysates.

269 In conclusion, ingested IP6 may be partially digested by phytase from foods
270 or intestinal bacteria. In this study, we demonstrated that hydrolysates of IP6 were able
271 to suppress HCT116 colon carcinoma cells as well as IP6. However, IP6 hydrolysates
272 suppress cell proliferation through an obviously different mechanism from that of intact
273 IP6, suggesting that partially degraded IP6 products are responsible for the suppression
274 of colon carcinogenesis by IP6.

275

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278

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345

346 **Figure legends**

347 Fig. 1. Changes in number of HCT116 cells after exposure to IP6 (a) or IP3-RPH (b) for
348 48 h. SEMs are shown as error bars (n = 6). Values not sharing the same letter are
349 significantly different, $P < 0.05$.

350

351 Fig. 2. Representative plots (left panels) of propidium iodide (PI) incorporation and the
352 distributions (right panels) in cell cycle of HCT116 cells cultured with 0.3 mM (a), 0.75
353 mM (b), or 1.5 mM (c) of IP6 (solid lines in left panels and filled bars in right panels)
354 compared to control (dashed lines in left panels and open bars in right panels) for 48 h.
355 Values in the cells cultured without IP6 are shown as open bars. SEMs are shown as
356 error bars (n = 6). Values with asterisk are significantly different from the value in
357 control (open bar), $P < 0.05$.

358

359 Fig. 3. Representative plots (left panels) of propidium iodide (PI) incorporation and the
360 distributions (right panels) in cell cycle of HCT116 cells cultured with 0.3 mM (a), 0.75
361 mM (b), or 1.5 mM (c) of IP3-RPH (solid lines in left panels and filled bars in right
362 panels) compared to control (dashed lines in left panels and open bars in right panels)
363 for 48 h. Values in the cells cultured without IP3-RPH are shown as open bars. SEMs
364 are shown as error bars (n = 6). Values with asterisk are significantly different from the
365 value in control (open bar), $P < 0.05$.

366

Fig. 4. mRNA expression of cyclin D1 (a), cyclin D3 (b), cyclin E2 (c), and cyclin A2 (d) in HCT116 cells exposed to IP6 or IP3-RPH for 48 h. Data was expressed as the relative band density of each cyclin against GAPDH. SEMs are shown as error bars (n = 6). Values not sharing the same letter are significantly different, $P < 0.05$.

Fig. 5. Representative plots of BrdU and propidium iodide (PI) staining in HCT116 cells exposed to 0 mM (a), 0.3 mM (b), 0.75 mM (c), and 1.5 mM (d) of IP3-RPH for 48 h. (e) Proportion of the BrdU incorporating HCT116 cells are shown in the indicated concentrations of IP3-RPH. SEMs are shown as error bars (n = 6). Values not sharing the same letter are significantly different, $P < 0.05$.

Fig. 6. Changes in number of IEC6 cells after exposure to IP6 or IP3-RPH for 48 h (a). SEMs are shown as error bars (n = 6). Values not sharing the same letter are significantly different, $P < 0.05$. Representative plots of propidium iodide (PI) incorporation in IEC6 cells cultured with vehicle medium (b), the medium containing 1.5 mM of IP6 (c), or the medium containing 1.5 mM of IP3-RPH (d) for 48 h.

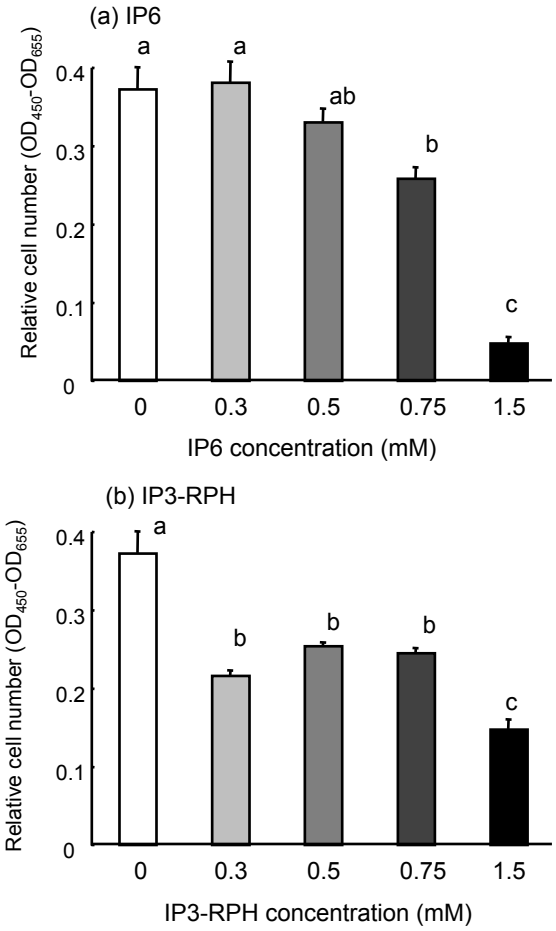

(a) IP6
a
a
ab
b
c
Relative cell number (OD450-OD655)
0.4
0.3
0.2
0.1
0
0
0.3
0.5
0.75
1.5
IP6 concentration (mM)
(b) IP3-RPH
a
b
b
b
c
Relative cell number (OD450-OD655)
0.4
0.3
0.2
0.1
0
0
0.3
0.5
0.75
1.5
IP3-RPH concentration (mM)

Fig. 1

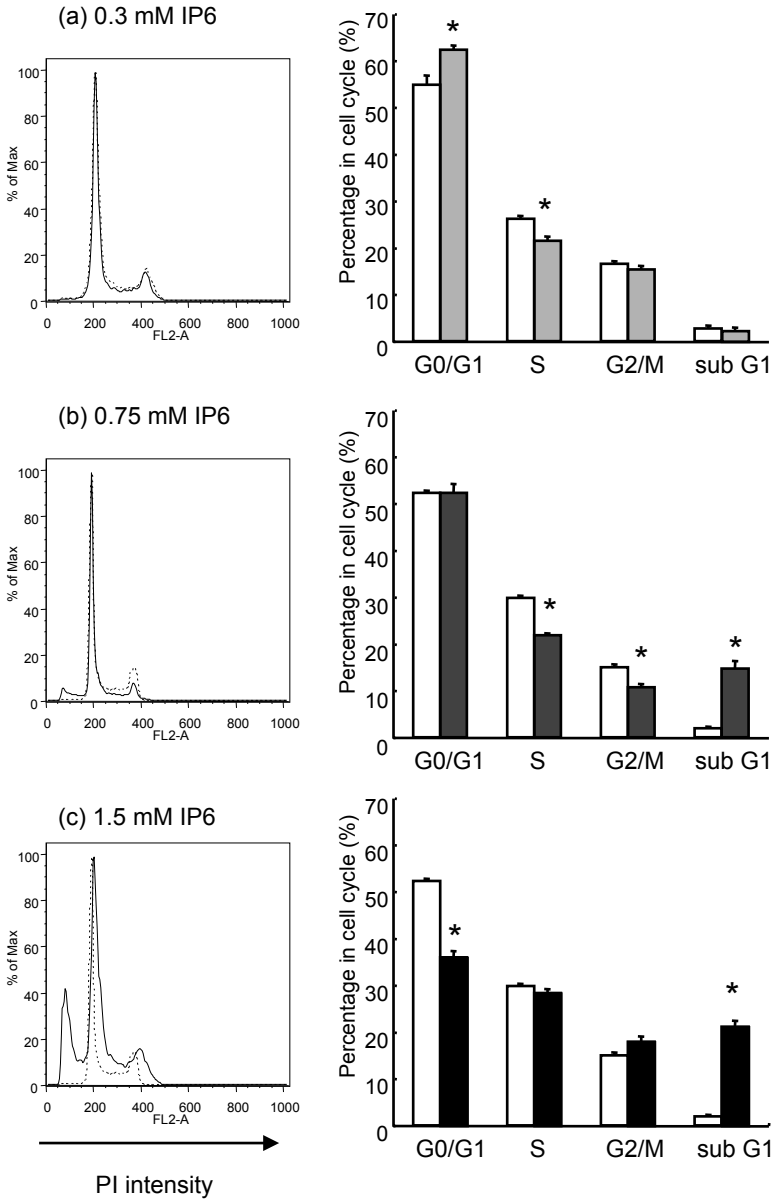

(a) 0.3 mM IP6
(b) 0.75 mM IP6
(c) 1.5 mM IP6
% of Max
FL2-A
Percentage in cell cycle (%)
G0/G1
S
G2/M
sub G1
PI intensity

Fig. 2

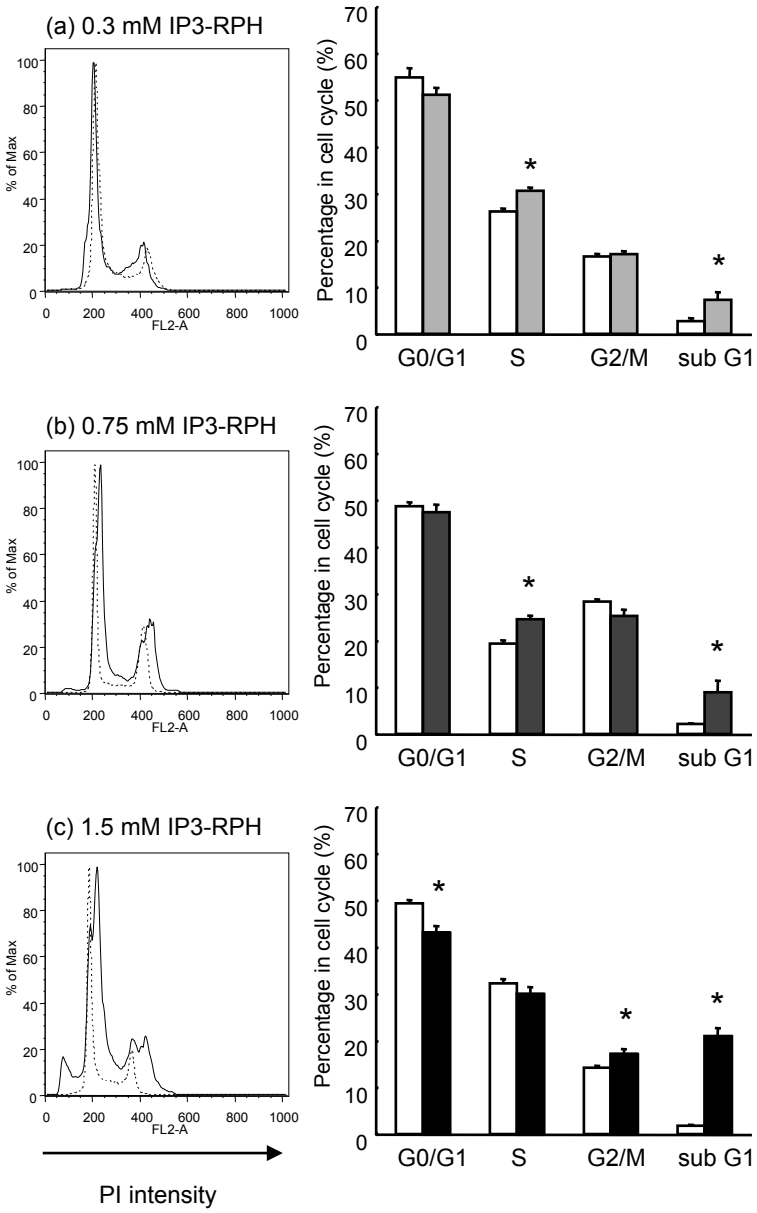

(a) 0.3 mM IP3-RPH
(b) 0.75 mM IP3-RPH
(c) 1.5 mM IP3-RPH
% of Max
FL2-A
Percentage in cell cycle (%)
G0/G1
S
G2/M
sub G1
PI intensity

Fig. 3

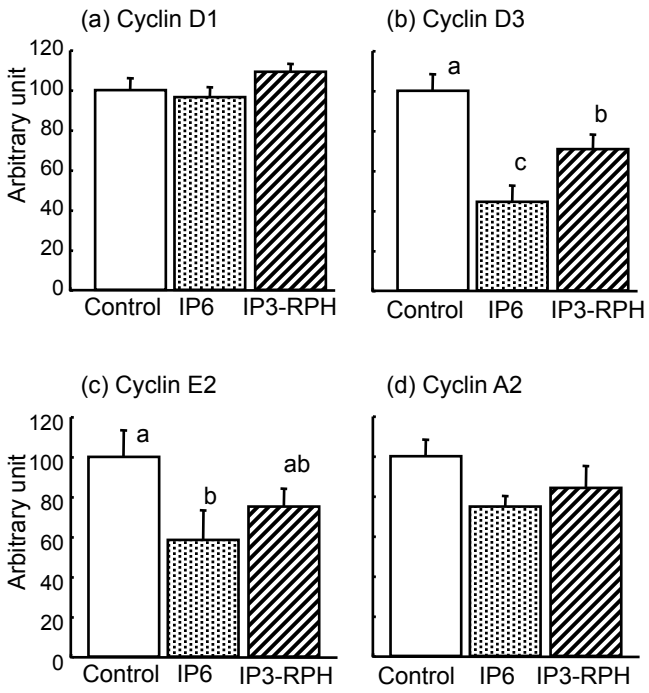
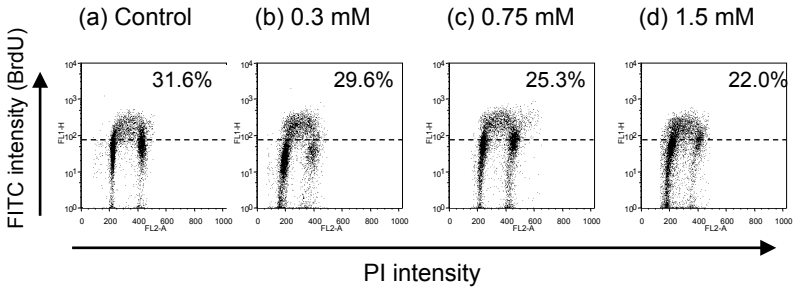

(a) Cyclin D1
(b) Cyclin D3
(c) Cyclin E2
(d) Cyclin A2
Arbitrary unit
120
100
80
60
40
20
0
Control
IP6
IP3-RPH
a
b
c
ab

Fig. 4


(a) Control
(b) 0.3 mM
(c) 0.75 mM
(d) 1.5 mM
31.6%
29.6%
25.3%
22.0%
FITC intensity (BrdU)
PI intensity
FL1-H
FL2-A

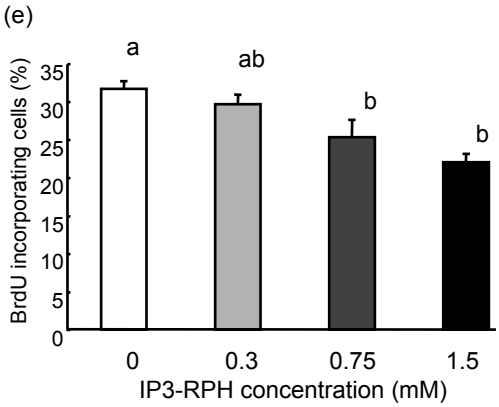

(e)
a
ab
b
b
BrdU incorporating cells (%)
0
5
10
15
20
25
30
35
0
0.3
0.75
1.5
IP3-RPH concentration (mM)

Fig. 5

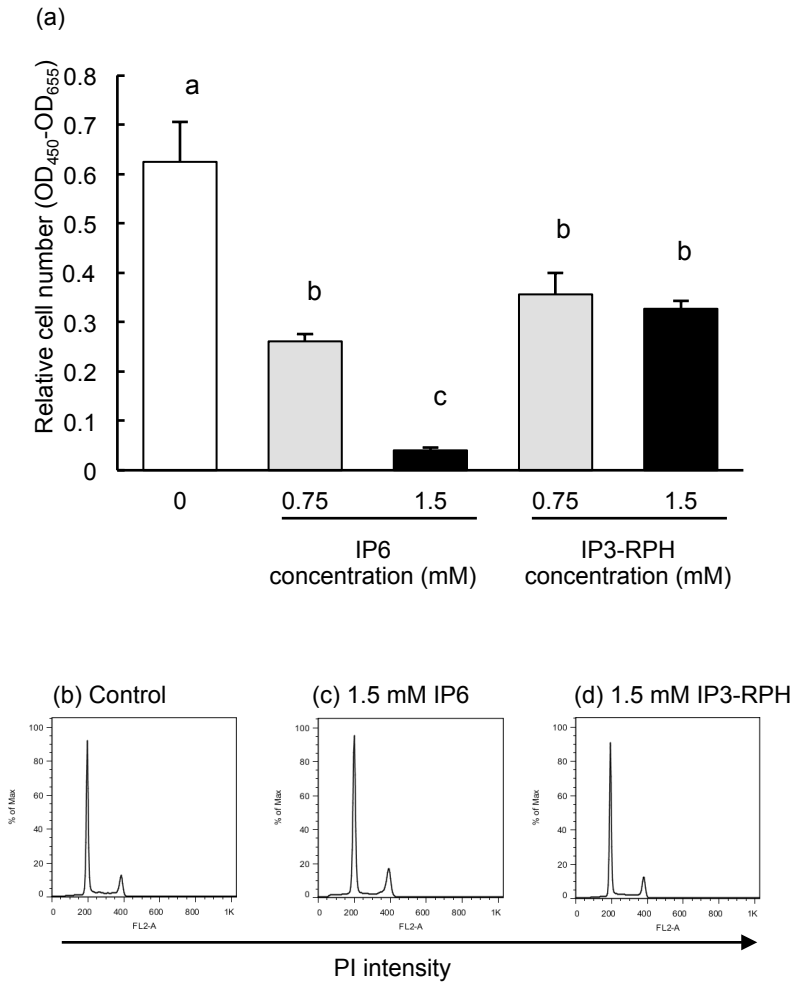


Fig. 6. Ishizuka *et al*.