EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 36, No. 1, 78-84, February 2004

Methyl-β-cyclodextrin inhibits cell growth and cell cycle arrest *via* a prostaglandin E_2 independent pathway

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Accepted 12 January 2004

Abbreviation: COX, cyclooxygenase; EIA, enzyme immuno assay; PG, prostaglandin

Abstract

Methyl-β-cyclodextrin, a cyclic oligosaccharide known for its interaction with the plasma membrane induces several events in cells including cell growth and anti-tumor activity. In this study, we have investigated the possible role of cyclooxygenase 2 (COX-2) in cell growth arrest induced by methyl-β-cyclodextrin in Raw264.7 macrophage cells. Methyl-β-cyclodextrin inhibited cell growth and arrested the cell cycle, and this cell cycle arrest reduced the population of cells in the S phase, and concomitantly reduced cyclin A and D expressions. Methyl-β-cyclodextrin in a dose- and time-dependent manner, also induced COX-2 expression, prostaglandin E_2 (PGE_2) synthesis, and COX-2 promoter activity. Pretreatment of cells with NS398, a COX-2 specific inhibitor completely blocked PGE_2 synthesis induced by methyl-β-cyclodextrin, however inhibition on cell proliferation and cell cycle arrest was not effected, suggesting non-association of COX-2 in the cell cycle arrest. These results suggest that methyl-β-cyclodextrin induced cell growth inhibition and cell cycle arrest in Raw264.7 cells may be mediated by cyclin A and D1 expression.

Keywords: cyclin A; cyclin D1; cyclooxygenase-2; methyl-β-cyclodextrin

Introduction

Cyclodextrins are known for their ability to modify the physico-pharmaceutical properties of various drugs and components through inclusion complex formation. This inclusion of the drug may have several advantages, *i.e*., increased aqueous solubility and stability, and a reduction in undesirable side effects (Loftsson *et al*., 2001). Methyl-β-cylodextrin is able to potentiate the *in vitro* anti-tumoral activity of doxorubicin in several sensitive cell lines (Grosse *et al*., 1998). Several studies have shown some cyclodextrins, including methyl-β-cyclodextrin, have unique cellular activities in terms of its interaction with the plasma membrane, permeabilization or hemolytic activity (Hirayama *et al*., 1990; Loftsson *et al*., 2002; Nagase *et al*., 2002). Methyl-β-cyclodextrin is considered as an interesting candidate for experimental cancer treatment because of its relatively low toxicity, unlike di- and tri-methyl-β-cyclodextrin (Grosse *et al*., 1999).

Cyclooxygenase (COX) catalyzes the first rate-limiting step in the formation of prostaglandin and thromboxane eicosanoids from phospholipase A_2 released arachidonic acid (van der Donk *et al.,* 2002). It is also known as prostaglandin endoperoxide H synthase, COX is responsible for both the cyclooxygenase reaction, in which arachidonic acid is converted to prostaglandin G_2 (PGG_2), and the peroxidase reaction, in which this intermediate undergoes a bi-electron reduction to PGH_2 (Kiefer *et al*., 2000). Two forms of COX have been identified, which are encoded by distinct genes, but which exhibit structural and enzymatic similarities. COX-2 is normally not expressed in cells, but is rapidly induced to produce in response to a wide variety of cytokines, and growth factors. COX-2 is an important target in the treatment of arthritis, pain, and possibly cancer (Turini *et al*., 2002). Moreover, the expression of COX-2 is increased in inflamed tissues such as, the rheumatoid synovium, and selective COX-2 inhibitors are useful for the treatment of arthritis (Crofford, 1999). COX-2 is also overexpressed in transformed cells and in tumors (Dannenberg *et al*., 2001). Although chemopreventive strategies have focused on inhibitors of COX enzyme activity, the mechanism of COX-2 inhibition with anticancer activity is still well understood (Mestre *et al*., 1999). Moreover, selective COX-2 inhibitors have been found to prevent the formation of intestinal and skin tumors in experimental animals (Pentland *et al*., 1999). Thus, it is reasonable to pos-

tulate that compounds, which induce COX-2 expression could predispose neoplsia.

COX-derived PGE_2 have been shown to exert many biological properties such as the induction of inflammation, the maintenance of kidney function and the modulation of the immune response (DuBoise *et al.*, 1998). Moreover, PGE_2 can modulate the proliferation of cells including fibroblasts, macrophages and some types of malignant cells (Harris *et al.,* 2002). Earlier studies have suggested that PGE_2 can promote the proliferation in various cell types including glandular epithelial cell, colorectal carcinoma cell (Sheng *et al.*, 2001; Buchanan *et al.*, 2003). However, other reports showed anti-proliferative action of PGE_2 (Belvisi *et al.*, 1998; Keerthisingam *et al.*, 2001).

The present study was undertaken to assess the effect of methyl-β-cyclodextrin on the growth of cells and their production of PGE_2, and to determine the relationship between cell growth and PGE_2 production in macrophages. We found that methyl-β-cyclodextrin induced cell growth inhibition and the cell cycle arrest *via* cyclin A and D regulation and in a dose- and time-dependent manner, stimulated the expression of COX-2 protein, and PGE_2 production independent of cell cycle arrest and cell growth inhibition effect.

Materials and Methods

Materials and cell culture

Methyl-β-cyclodextrin and PI were obtained Sigma-Aldrich (St. Louis, MO); NS398 was from BioMol (Plymouth Meeting, PA); [^{3}H]thymidine and the PGE_2 enzyme immunoassay (EIA) kit from Amersham Pharmacia Biotech (Piscataway, NJ). For Western blot analysis, we used a rabbit antibody (Ab) against COX-2 (Cayman Chemicals, Ann Arbor, MI) and cyclin A, D1, D3 and cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase-conjugated anti rabbit IgG, or anti-mouse IgG were used as secondary antibodies. The macrophage cell line (Raw264.7) was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI medium (Life Technologies Inc.) supplemented with 10% fetal calf serum (Hyclone) and an antimycotic/antibiotic mixture (Life Technologies, Inc.).

Cell number counting and thymidine uptake measurement

Raw264.7 cells were grown in 12-well plates at a concentration of 5×10^5 cells/well, and then [^{3}H]thymidine (0.4 μCi/well) was added for 4 h. The plates were washed with PBS 12 h later, and scintillation fluid was added for the DNA synthesis assay and radioactivity quantified in a scintillation counter. For counting viable cell numbers, trypan blue was added to the cells at a 1:1 dilution, and cells were counted using a hemocytometer.

Determination of PGE_2 production

PGE_2 production was measured using a commercial PGE_2 enzyme immunoassay (EIA) kit. Cells were cultured in 6-well plates and stimulated with methyl-β-cyclodextrin. Supernatant samples were obtained at the indicated doses or times and subjected to EIA analysis.

Transfection and promoter activity assay

The COX-2 promoter DNA with full-length (-1432/+59 bp) was constructed into pGL2 vector. Cells were transfected with COX-2 promoter DNA using Lipofectamine 2000. After transfection, cells were incubated in complete media for 24 h at 37°C before being stimulated with methyl-β-cyclodextrin for 12 h at 37°C. Cell lysates were assayed for luciferase activity according to the manufacture's instructions (Promega) using a luminometer.

Protein extraction and Western blot analysis

Cells were washed twice in cold PBS and lysed on ice using lysis solution (1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail). Sample protein concentrations were determined using the Bio-Rad protein assay. Proteins from the cell lysates (30 μg) were boiled at 95°C in Laemmli SDS loading buffer, separated on 8% SDS-PAGE, and electrotransferred to nitrocellulose membranes. The membranes were then blocked for at least 30 min at room temperature in Tris buffered saline-0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing the primary antibody. For immunoblotting of COX-2, incubated for 4 h at room temperature. After 5 washes of 30 min each in TTBS, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h. After 5×30 min washes with TTBS, enhanced chemiluminescence detection (Amersham) was performed and the membranes were exposed to X-ray films.

Flow cytometry cell cycle analysis

After methyl-β-cyclodextrin treatment, cells were washed with PBS, trypsinized, fixed with 70% ethanol, and stored at -20°C. Cells were then stained for 30 min at room temperature with a 20 μg/ml PI solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analyzed on a FACS flow cytometer (Becton Dickenson, Mountain

View, CA). At least 10,000 fluorescent cells were counted per sample.

Results

Effects of methyl-β-cyclodextrin on cell growth

In order to measure Raw264.7 macrophages growth following treatment with varying levels of methyl-β-cyclodextrin, both cell counts and [^{3}H]-thymidine uptake into cells were used. A close correlation was observed between cell count and [^{3}H]thymidine uptake experiments. Methyl-β-cyclodextrin at doses of less than 1 mM did not reduce macrophage cell growth. In the absence of other cofactors, increasing concentrations of methyl-β-cyclodextrin above 1 mM resulted in a significant decrease in cell growth in a dose-dependent manner. Methyl-β-cyclodextrin caused inhi-

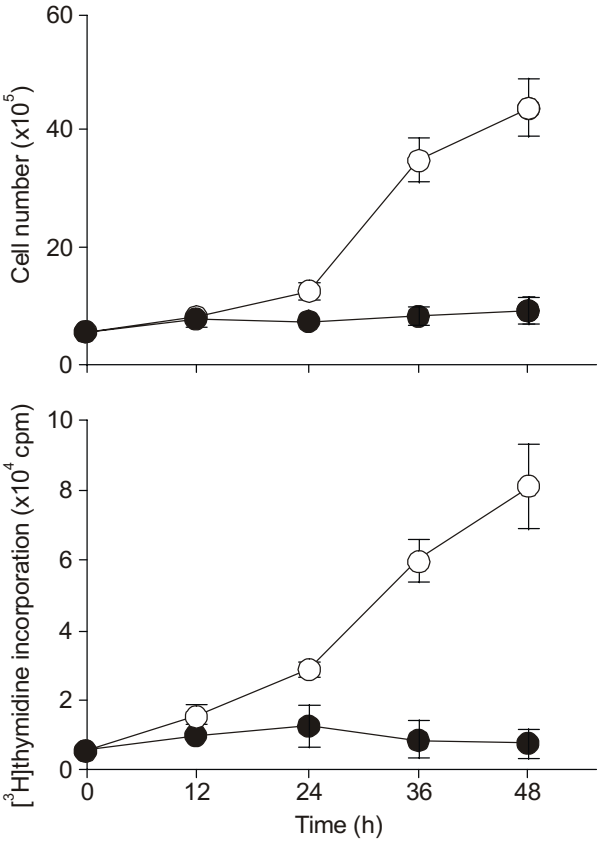

Figure 1. Effect of methyl-β-cyclodextrin on the cell growth of Raw264.7 cells. Time-dependent effect of methyl-β-cyclodextrin on cell growth evaluated by trypan blue dye exclusion and [^{3}H]thymidine incorporation into DNA. [^{3}H]thymidine was incubated for 4 h before end of the reaction time. Cells were cultured for the indicated times in the presence (●) or absence (○) of 6 mM methyl-β-cyclodextrin. Data represents the means±SD of 5 independent experiments performed in duplicate.

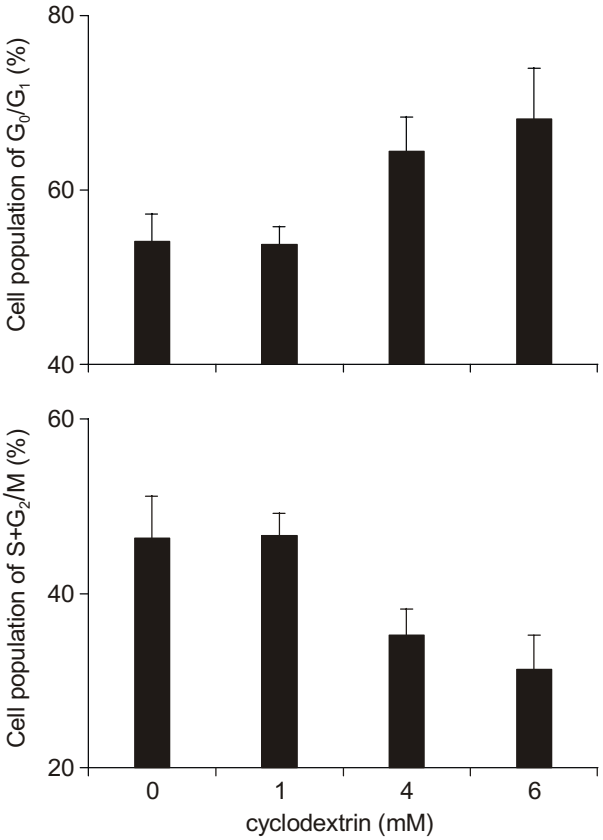

Figure 2. Effect of methyl-β-cyclodextrin on the cell cycle of Raw264.7 cells. Raw264.7 cells were untreated or treated with methyl-β-cyclodextrin for the indicated times. Fixed cells were incubated with RNase A, stained with propidium iodide, and subjected to FACScan analysis to determine the distribution of cells through the G1, S and G$_2$/M phases. Percentages of cells in the G0/G1 or S+G$_2$/M phases in the control and in methyl-β-cyclodextrin-treated cultures.

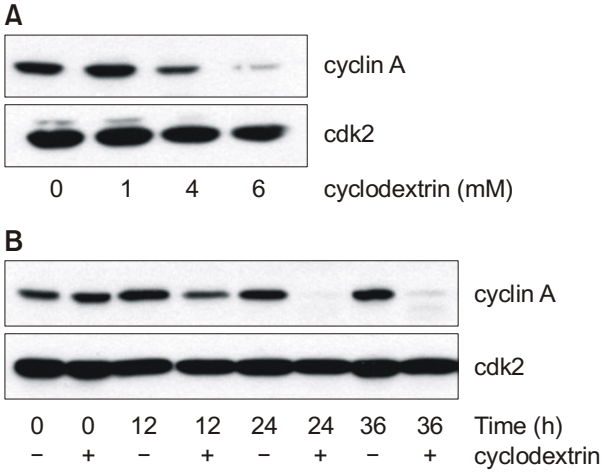

Figure 3. Effect of methyl-β-cyclodextrin on the expressions of cyclin A and cdk2. Cells were treated with methyl-β-cyclodextrin at the indicated doses (A) or times (B), and the expressions of cyclin A and cdk2 proteins were analyzed by Western blot, as described in Materials and Methods.

bition of cell growth after 12 h treatment and the growth inhibition was sustained (Figure 1).

For the analysis of cell cycle distribution affected by methyl-β-cyclodextrin treatment, the treated cells was first stained with propidium iodide and FACS analysis was carried out. Methyl-β-cyclodextrin-treated cells were associated with a significant decrease in the S+G_2M phase population and an increase in the G0/G1 population in comparison with those of untreated cells in a dose-dependent manner (Figure 2).

To determine whether these changes in cell cycle arrest were related to expressional differences of cell cycle regulatory proteins, Western blotting of cell lysates was carried out using the antibodies of various cell cycle-related proteins. Among these, cyclin A was found to be reduced by methyl-β-cyclodextrin treatment in a dose- and time-dependent manner (Figure 3). In addition, the expressions of cyclin D1 and D3 were also reduced significantly by methyl-β-cyclodextrin (data not shown). However, the expression of the cdk2 was unaffected by methyl-β-cyclodextrin treatment.

Methyl-β-cyclodextrin induces COX-2 expression and PGE_2 production in Raw264.7 cells

The possible effects of methyl-β-cyclodextrin on PGE_2 production in Raw264.7 cells were examined by EIA method. The data in Figure 4A and B shows that methyl-β-cyclodextrin caused dose- and time-dependent increases in PGE_2 production. These changes in PGE_2 synthesis were accompanied by increased expression of COX-2, as shown in the Western blotting of cell lysates (Figure 4A and B). Neither COX-1 nor hsp70 expression was altered by methyl-β-cyclodextrin. Thus, the effects of methyl-β-cyclodextrin on

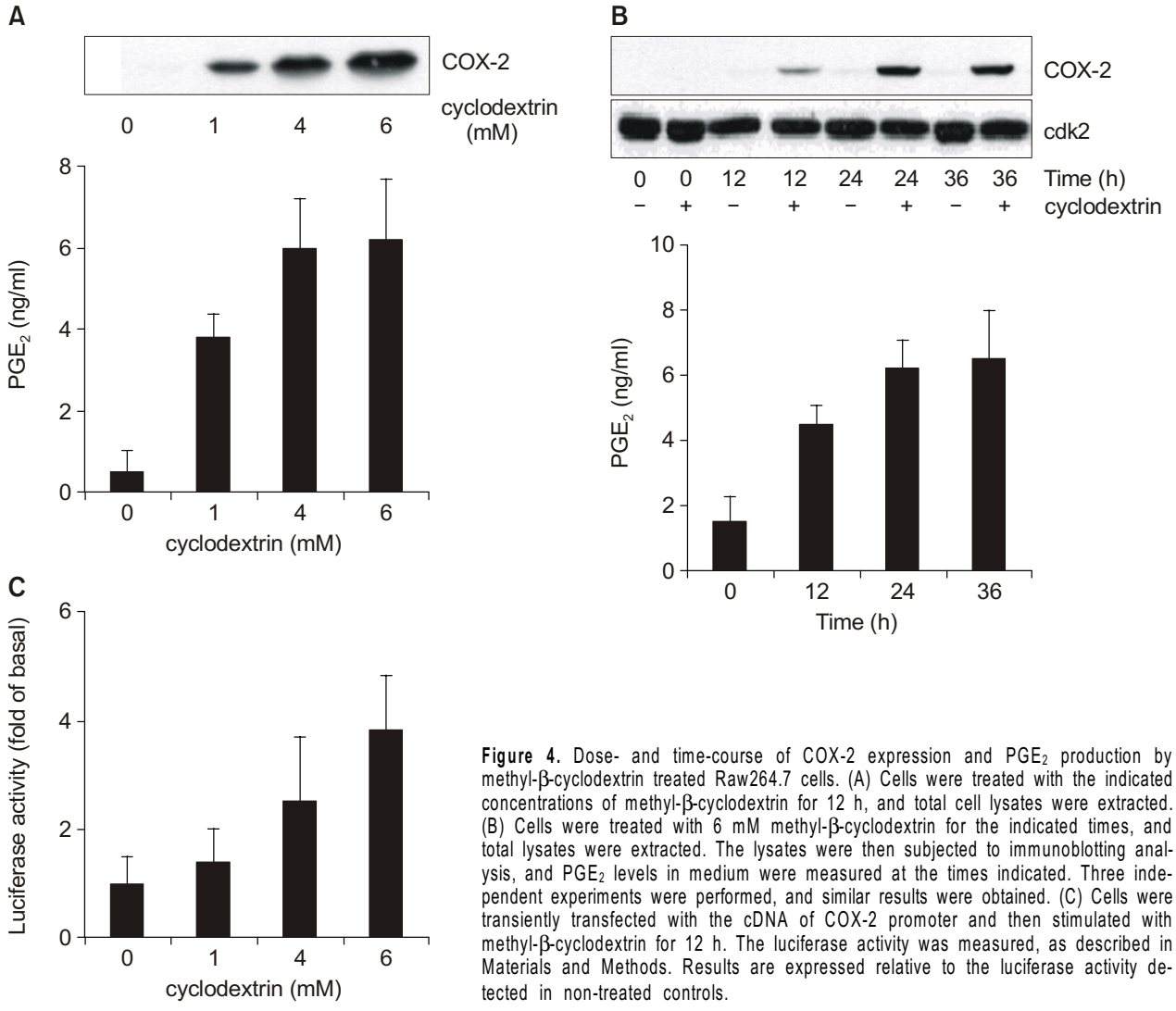

Figure 4. Dose- and time-course of COX-2 expression and PGE_2 production by methyl-β-cyclodextrin treated Raw264.7 cells. (A) Cells were treated with the indicated concentrations of methyl-β-cyclodextrin for 12 h, and total cell lysates were extracted. (B) Cells were treated with 6 mM methyl-β-cyclodextrin for the indicated times, and total lysates were extracted. The lysates were then subjected to immunoblotting analysis, and PGE_2 levels in medium were measured at the times indicated. Three independent experiments were performed, and similar results were obtained. (C) Cells were transiently transfected with the cDNA of COX-2 promoter and then stimulated with methyl-β-cyclodextrin for 12 h. The luciferase activity was measured, as described in Materials and Methods. Results are expressed relative to the luciferase activity detected in non-treated controls.

PGE_2 synthesis were definitively mediated by COX-2.

To pursue a path of COX-2 regulation by methyl-β-cyclodextrin treatment on cells, the effect on COX-2 transcription were explored. Cells were transfected with a reporter plasmid, in which luciferase was driven by the COX-2 promoter. As shown in Figure 4C, transfection of COX-2 promoter induced a marked concentration-dependent increase in activity. The maximal increase in relative luciferase activity was 4-fold in methyl-β-cyclodextrin treated cells versus controls. To confirm that methyl-β-cyclodextrin regulated COX-2 transcriptional mechanism, we treated the cells with methyl-β-cyclodextrin after pretreating with actinomycin D for 1 h. Actinomycin D, an inhibitor of RNA synthesis, suppressed the COX-2 expression induced by methyl-β-cyclodextrin.

A PGE_2-independent mechanism is involved in cell growth arrest by COX-2

The down-regulation of cyclin A and D proteins was found to be closely correlated with methyl-β-cyclodextrin growth inhibition in Raw264.7 cells. To further explore-induced the involvement of PGE_2 in methyl-β-cyclodextrin-induced growth inhibition of macrophages expressing COX-2 protein, we examined the kinetics of the system with respect to cell number, cyclin expression, and cell cycle after cells had been exposed to NS398 or indomethacin. As shown in Figure 5, the pretreatment of cells with 10 μM of NS398, a COX-2 specific inhibitor did not affect cell growth inhibition induced by the methyl-β-cyclodextrin but resulted in the near complete inhibition of PGE_2 synthesis. In addition, the attenuation of methyl-β-cyclodextrin-induced cyclin A expression and of the cell cycle was

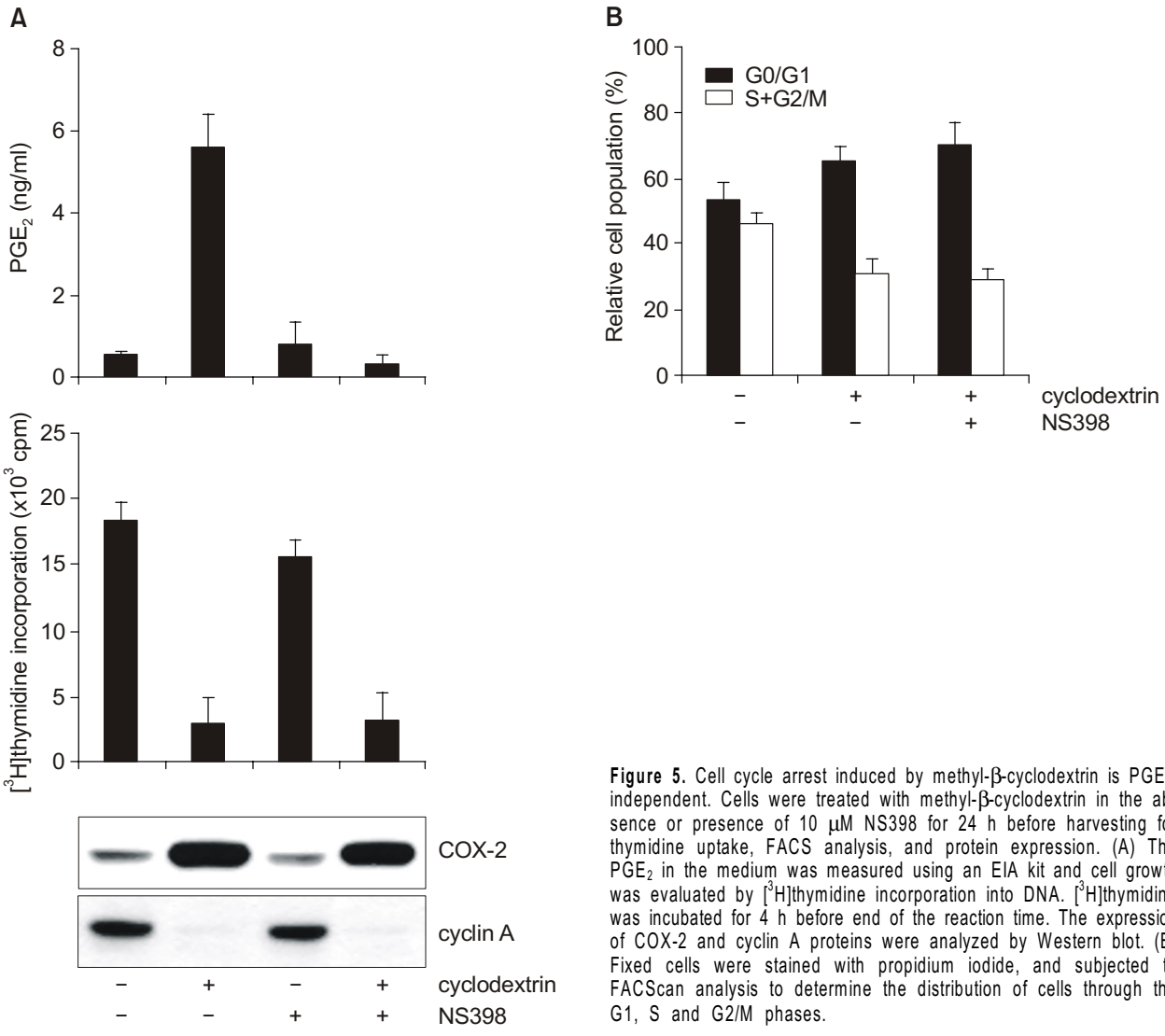

Figure 5. Cell cycle arrest induced by methyl-β-cyclodextrin is PGE_2-independent. Cells were treated with methyl-β-cyclodextrin in the absence or presence of 10 μM NS398 for 24 h before harvesting for thymidine uptake, FACS analysis, and protein expression. (A) The PGE_2 in the medium was measured using an EIA kit and cell growth was evaluated by $[^3H]$thymidine incorporation into DNA. $[^3H]$thymidine was incubated for 4 h before end of the reaction time. The expression of COX-2 and cyclin A proteins were analyzed by Western blot. (B) Fixed cells were stained with propidium iodide, and subjected to FACScan analysis to determine the distribution of cells through the G1, S and G2/M phases.

not recovered by pretreating cells with NS398.

Discussion

In the present study, methyl-β-cyclodextrin was found to induce G1 growth arrest in macrophages associated with an inhibition in the expression of cyclin A. In addition, methyl-β-cyclodextrin induces COX-2 expression and PGE_2 production. These findings are consistent with the previous reports that cyclin A plays a major role in the cell growth. However, methyl-β-cyclodextrin-mediated growth arrest does not require the synthesis of PGE_2.

It has previously been reported that methyl-β-cyclodextrin significantly inhibits cell growth (Grosse *et al*., 1998), although the detailed mechanisms of this growth inhibition are unclear. To elucidate the molecular mechanism of growth inhibition by methyl-β-cyclodextrin, both cell number and [^{3}H]thymidine incorporation experiment were carried out and assuredly established that this molecule caused arrest of cell growth. In addition, to investigate whether cell cycle arrest plays a role in the anti-proliferative effects of methyl-β-cyclodextrin, Raw264.7 cells were subjected to FACS analysis after methyl-β-cyclodextrin treatment. Alterations in the cell growth of Raw264.7 cells in response to methyl-β-cyclodextrin treatment were found to be associated with changes in the G1 and S phases of cell cycle. Methyl-β-cyclodextrin-treated macrophages accumulated at G1, and showed reduced [^{3}H]thymidine incorporation. A clear anti-proliferative effect of methyl-β-cyclodextrin shown in Raw264.7 cells is likely associated with its affinity for cell membrane lipid components, particularly cholesterol, which plays a major role in the structure and function of the cell membrane (Jadot *et al*., 2001; Steck *et al*., 2002). Also methyl-β-cyclodextrin, a cyclic oligosaccharide is known to interact with the lipid components of biological membranes, and to modulate their fluidities and permeabilities (Hartel *et al*., 1998).

Cyclins control the transitions between the phases of the cell cycle as regulatory subunits of the cdks. Cyclin A levels increase at the G1-S boundary and reach peak levels in S phase (Pines, 1993; Hong *et al*., 2002). Work by several investigators has also demonstrated that the genes of D-type cyclins regulate the rate of progression through G1 (Sherr, 1995; Coqueret, 2002). Moreover, the inhibition of cell growth has been associated with the down-regulation of cyclin D1, with little effect on cdk4 or cyclin D3 levels (Trifan *et al*., 1999). Cyclin A and D were critical targets of proliferative signals following the growth factor stimulation of quiescent cells, and their inhibition can cause a G1 arrest (Kim *et al*., 2003). Our results demonstrate that cyclin A and D1 protein levels are significantly attenuated in methyl-β-cyclodextrin-treated cells, whereas the cdks levels, including those of cdk2 and cdk4, did not change in cells. Therefore, cyclin A and D1 seem to perform a crucial role in regulating the progression of macrophages through the first gap of the cell cycle. These results can be drawn to conclude that cyclin A and D1 expressions have a major role in the cell cycle arrest triggered by methyl-β- cyclodextrin.

Methyl-β-cyclodextrin induces COX-2 expression in macrophages. It remains to be determined whether growth arrest by methyl-β-cyclodextrin was linked to COX-2 expression and PGE_2 production. To assess the specificity of G1 arrest subsequent to the overexpression of COX-2, a control experiment was carried out by overexpressing COX-2 protein in HEK293 cells. When COX-2 was overexpressed in the HEK293 cells, cell growth was decreased compare to that of the vector transfected control cells (data not shown). From these results, COX-2 product PGE_2 may not be ruled out in its effect on the cell growth. Therefore, we next determined whether PGE_2 secretion by COX-2 is necessary for the cell cycle arrest caused by methyl-β-cyclodextrin. The COX-2 inhibitor, NS398 treatment resulted in complete inhibition of PGE_2 secretion by methyl-β-cyclodextrin-treated cells. However, pretreatment of cells with NS398 did not affect cell cycle arrest or cyclin A down-regulation by methyl-β-cyclodextrin. These results suggest that PGE_2 is not required in the methyl-β-cyclodextrin-mediated cell cycle arrest. Generally, the expression of COX-2 is associated with a variety of proliferative diseases, such as rheumatoid arthritis, colorectal cancer, and gastric cancer (Dubois *et al*., 1998; Zha *et al*., 2001). In addition, it is generally accepted that the COX-2 expression leads to the secretion of prostanoids including PGE_2. Trifan *et al.* (1999) reported that interactions between secreted prostanoids and membrane receptors trigger proliferative effects and the sustained overexpression of COX-2 induces growth arrest. However, interestingly, the ability of methyl-β-cyclodextrin to induce growth arrest is independent of PGE_2 secretion. This is consistent with the fact that interleukin-1 and PMA-induced endothelial cell growth arrest is not reversed by the nonsteroidal anti-inflammatory drugs that block prostanoid synthesis (Ristimaki et *al*., 1994) and with the finding that COX-2 overexpression induces G1 growth arrest by a hitherto uncharacterized nonprostanoid-dependent signaling pathway (Trifan *et al*., 1999). Further studies are required to define this novel mechanism of COX-2 function at a molecular level, and to assess its physiological relevance.

Acknowledgement

This work was supported by research grant of Advanced Research Center in Yeungnam University.

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