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INFLUENCE OF INOSITOL HEXAPHOSPHATE ON THE EXPRESSION OF SELECTED PROLIFERATION MARKERS IN IL-1β-STIMULATED INTESTINAL EPITHELIAL CELLS

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Abstract: The aim of the present study was to examine the influence of IP6, a naturally occurring phytochemical, on the expression of genes coding for proliferation markers, i.e., cyclin D1 (CCND1) and histone H3 in IL-1β-stimulated intestinal cancer cell line Caco-2. Quantification of genes expression was carried out using real time RT-QPCR technique in Caco-2 cells after treatment with IL-1β, 1 and 2.5 mM of IP6 for 3, 6 and 12 h. In separate cultures, cells were incubated with IL-1β for the indicated times. The untreated Caco-2 cells were used as the control. In a time course experiment, stimulation of cells with IL-1β only resulted in an overexpression of both CCND1 and histone H3 mRNAs as compared with control. IP6 had no influence on IL-1β-stimulated CCND1 expression for 3 and 6 h. After 12 h, statistically significant decrease in CCND1 mRNA was observed in cells exposed to IL-1β and IP6 (1 and 2.5 mM) in relation to cells treated with IL-1β only. The levels of H3 mRNA in IL-1β-stimulated cells and cells treated with IL-1β and IP6 revealed no statistically significant differences after 3 h. IP6 at 1 and 2.5 mM enhanced IL1β-stimulated transcription of H3 gene after 6 h. Subsequently (12 h), the combination of IP6 and IL-1β decreased H3 mRNA level compared to IL1β-treated cells. In conclusion, pro-inflammatory cytokine IL-1β up-regulates CCND1 and histone H3 mRNAs expression in Caco-2 cells. These results suggest that the ability of IP6 to inhibit colon cancer cells proliferation may be mediated through downregulation of genes encoding cyclin D1 and histone H3 at the mRNA level.

Keywords: IP6, proliferation, histone H3; cyclin D1; colon cancer; mRNA quantification

The loss of cell proliferation control is one of the major features of malignancy. Proliferation rate serves as a useful prognostic factor for tumor cells survival and response to treatment with anticancer drugs (1) and it can be expressed as the fraction of cells residing in the S-phase (2). There are a few methods for detecting proliferating cells including flow cytometry, immunostaining of cell cycle-restricted proteins like proliferating cell nuclear antigen (PCNA) and Ki-67, and incorporation of thymidine analogues or bromodeoxyuridine (BrdU) into DNA (1). Proliferating cells can also be distinguished from postmitotic cells by the detection of RNAs expressed differentially during cell cycle (3). Cyclin D1 and histone H3 mRNA genes are the ones expressed in a cell-cycle related pattern.

Histone H3 is a part of the nucleosome, the fundamental repeating unit of chromatin (4). There are two classes of histone H3 proteins – first that is produced concomitantly with S-phase DNA synthesis and the second produced independently of DNA synthesis. Genes encoding the first class of H3 are called canonical H3 genes. Their products are either H3.1 or H3.2 protein variants, which harbor cysteine or serine at position 96. Cells residing outside the S-phase also need newly synthesized histones, which involves production of the H3.3 variant (5). Histone mRNA metabolism is tightly coupled to cell cycle progression. As cells progress from G1 to S-phase, the rate of histone gene transcription and histone pre-mRNA processing increases resulting in a 35-fold increase in histone mRNA level. After the S-phase histone mRNA level rapidly decrease (6). In several studies it was found out histone H3 mRNA to be a reliable and accurate marker of S-phase cells in both normal and tumor human tissues (1, 2, 7–11).

Cyclin D1 (CCND1), forming complexes with cyclin dependent kinases CDK4 and CDK6, functions as a regulator of G1 to S-phase progression.

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Control of this stage is a crucial checkpoint for the cell fate (12). CCND1 accumulation begins in G1-phase and acquires the highest level during G1/S-phase transition. In the S-phase CCND1 nuclear level rapidly declines due to its increased turnover and relocation to the cytoplasm (13). Induction of its expression results from stimulatory influence of extracellular mitogens, such as growth factors, cytokines and hormones (14). Thus, cyclin D1 overexpression and nuclear accumulation is implicated in many cancers (11, 15, 16) and is associated with increased cell proliferation (17).

Chronic inflammation has been linked to the pathogenesis of tumors in up to 15% of human cancers (18). One of the most prevalent relationship between inflammation and cancer is inflammatory bowel disease associated colorectal cancer. Tumor growth is contributed by various cytokines and chemokines produced by immune cells (19). Interleukin-1β plays a central role in the pathogenesis of inflammatory bowel disease and experimental intestinal inflammation. It is upregulated in many cancer types, as it has strong impact on the expression of growth and transcription factors, proliferative and angiogenic proteins and adhesion molecules (18).

Several naturally occurring compounds are known to have antiproliferative effects on cancer cells. One of them is inositol hexaphosphate, an abundant intrinsic component of high fiber diets, such as cereals, grains, seeds and legumes (20). Its antiproliferative action was established in *in vitro* and *in vivo* models of various neoplasms such as colon, prostate, pancreatic and breast cancer (21–26).

The aim of the present study was to examine the influence of IP6 on the expression of genes coding for proliferation markers, i.e., CCND1 and histone H3 in IL-1β-stimulated intestinal cell line Caco-2.

EXPERIMENTAL

Cell culture and cell stimulation assays

The Caco-2 human intestinal epithelial cells (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (GibcoBRL), 100 U/mL penicillin and 100 μg/mL streptomycin (both from Sigma Aldrich) and 10 mM HEPES (GibcoBRL). They were maintained at 37°C in a 5% CO_2 atmosphere. Cells were seeded into six-well plates (Nunc International) at a density of 4.5×10^5 per well and allowed to grow to confluency in 3 mL of medium. After three days, the culture media were changed to media with 2% FBS and cells were then cultured for 2 days. They were then stimulated with 1 ng/mL IL-1β (Sigma Aldrich) for 30 min. Afterwards, cells were treated with 1 and 2.5 mM IP6 as dipotassium salt (dissolved in distilled water and adjusted to pH 7.4) (Sigma Aldrich) for 3, 6 and 12 h. In separate cultures, cells were incubated with 1 ng/mL IL-1β for the indicated times. The untreated Caco-2 cells were used as the control.

Real-time RT-QPCR assay

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and was treated with DNase-I (Fermentas) to remove DNA contamination. RNA concentration was determined spectrophotometrically on the basis of absorbance values at a wavelength of 260 nm using a GeneQuant pro spectrophotometer (Amersham Biosciences). The expression of genes encoding CCND1 and histone H3 was detected using a real-time RT-QPCR technique with a SYBR Green chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen) and Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research). Oligonucleotide primers specific for CCND1 and H3 were previously described (11). The thermal profile for one-step RT-PCR was as follows: 50°C for 30 min for reverse transcription and 95°C for 15 min followed by 45 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 45 s for amplification. Specificity of RT-PCR reaction was confirmed by determining the characteristic temperature of melting for all amplimers. Each gene analysis was performed in triplicate. The mRNA copy numbers of examined genes were determined on the basis of the commercially available standard of β-actin (TaqMan DNA Template Reagent Kit, Applied Biosystems). The obtained results of mRNA copy number were recalculated per mg of total RNA. The expression level of genes in cultured cells was expressed as the fold change relative to the control. The value of fold change > 1 reflects increased expression of the target gene, and a value of fold change < 1 points to a decrease in the gene expression.

Statistical analysis

The results were collected from three independent experiments. Statistical analysis was performed with the use of Statistica PL 10.0 software. All the results are expressed as the means ± SD. Comparison of two data sets was performed by unpaired t-test. Comparison of more than two data sets was performed by one-way ANOVA followed

by *post-hoc* Tukey test. Significance level was assumed for $p < 0.05$.

RESULTS

Stimulation of Caco-2 with 1 ng/mL of IL-1β for 3–12 h resulted in an upregulation of CCND1 (Fig. 1A) and histone H3 (Fig. 2A) genes as compared with untreated cells ($p < 0.05$). In a time course experiment, the level of CCND1 mRNA in IL-1β-stimulated cultures remained relatively constant ($p = 0.171$) (Fig. 1A). Comparative analysis of H3 transcripts revealed statistically significantly diverse amounts in the cells treated with IL-1β for 3,

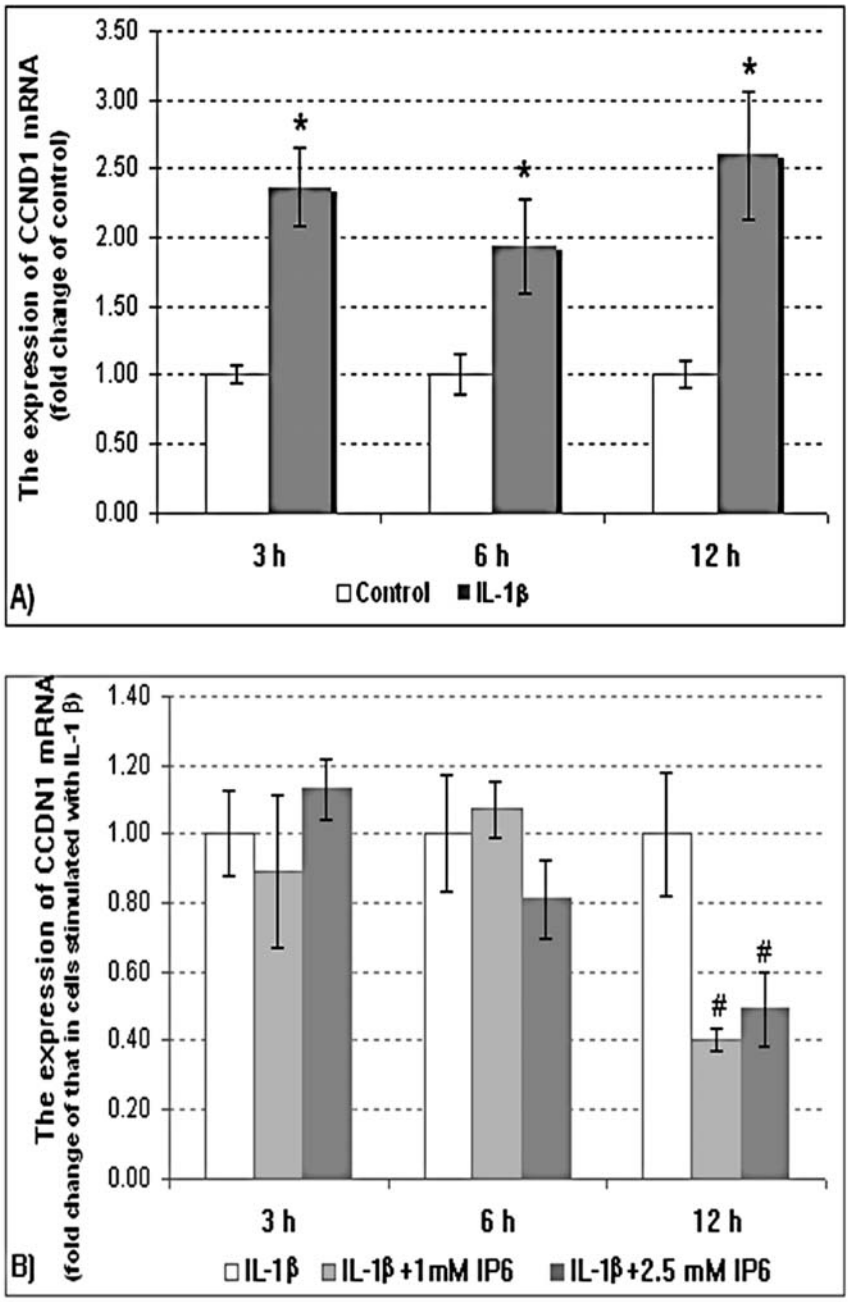

Figure 1. Expression of CCND1 gene in Caco-2 cells as determined by real-time RT-PCR. Changes in CCND1 mRNA expression in Caco-2 cells after treatment with A) IL-1β and B) IL-1β and 1 and 2,5 mM IP6 for 3, 6 and 12 h. The results are presented as the mean ± SD of three separate experiments; *$p < 0.05$ *vs.* control Caco-2 cells; #$p < 0.05$ *vs.* IL-1β-stimulated cells)

6 and 12 h ($p = 0.022$) (Fig. 2A). The highest level of this gene expression was determined in 3 h lasting cultures and subsequently the quantities of H3 mRNA were down-regulated within 3–12 h.

IP6 had no influence on IL-1β-stimulated CCND1 expression for both 3 and 6 h ($p > 0.05$). After 12 h, statistically significant decrease in CCND1 mRNA was observed in cells exposed to IL-1β and IP6 (1 and 2.5 mM) in relation to cells treated with IL-1β only ($p < 0.05$) (Fig. 1B).

The levels of H3 mRNA in IL-1β-stimulated cells and cells treated with IL-1β and IP6 revealed

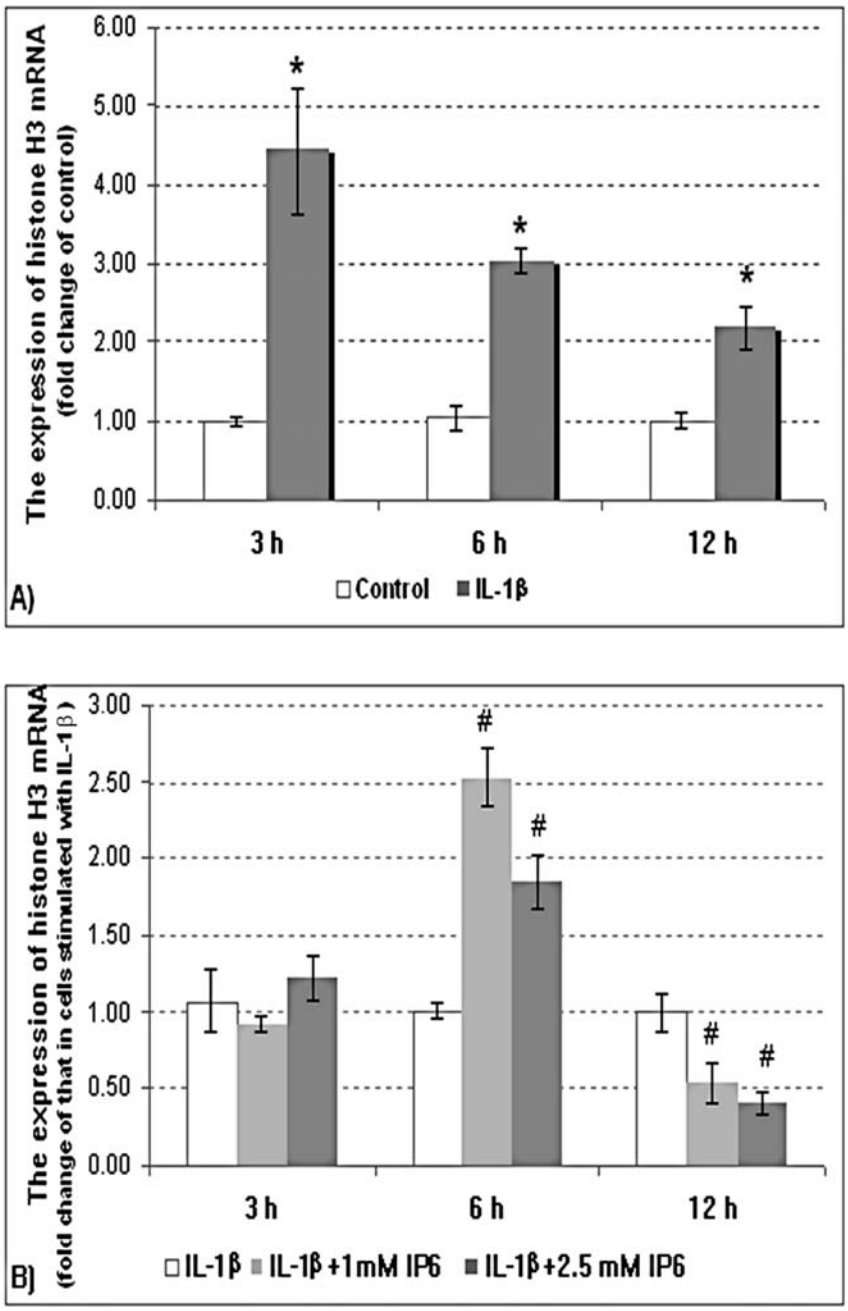

Figure 2. Expression of histone H3 gene in Caco-2 cells as determined by real-time RT-PCR. Changes in H3 mRNA expression in Caco-2 cells after treatment with A) IL-1β and B) IL-1β and 1 and 2,5 mM IP6 for 3, 6 and 12 h. The results are presented as the mean ± SD of three separate experiments; *$p < 0.05$ *vs.* control Caco-2 cells; #$p < 0.05$ *vs.* IL-1β-stimulated cells)

no statistically significant differences after 3 h. IP6 at a concentration of both 1 ($p < 0.001$) and 2.5 mM ($p = 0.003$) enhanced IL1β-stimulated transcription of H3 gene after 6 h and the stronger stimulatory effect was manifested by IP6 at lower concentration (1 mM). In longer-lasting cultures (12 h), transcriptional activity of this gene was suppressed about 2-fold by IP6 in a dose-independent manner in cells treated with IL-1β/IP6 as compared to those challenged with IL-1β alone ($p < 0.05$) (Fig. 2B).

DISCUSSION AND CONCLUSION

In the present study, the effect of IP6 on the expression of CCND1 and histone H3 genes in IL-1β-stimulated colon cancer cell line Caco-2 was evaluated. Colorectal cancer is one of the major causes of morbidity and mortality throughout the world, and its occurrence is frequently preceded by chronic inflammation. Several factors that are engaged in inflammatory response are also associated with increased proliferation of cells. Pro-inflammatory cytokines (INF-γ, TNF-α, IL-1 or IL-6) produced and secreted by macrophages, mast cells and neutrophils, may act as initiators and promoters of carcinogenesis by directly increasing the proliferation of epithelial cells (27). Our studies revealed that IL-1β induces transcription of both cyclin D1 and histone H3 genes in colon cancer cells. It is noteworthy that impact of this pro-inflammatory cytokine on histone H3 gene is most prominent for short-time incubation and diminishes in a time-dependent manner. The fact that both genes are known as proliferation markers indicates that IL-1 enhances cell proliferation. The exact mechanisms by which IL-1 promotes tumor growth is unclear, though it is known to contribute to activation of NF-κB and AP1 transcription factors (19). Both factors could be considered as mediators linking inflammation and cancer. These transcription factors have binding sequences in the promoter region of cyclin D1 gene (28). The results of the present study clearly support the connection between inflammation and enhanced proliferation.

According to Liao et al. (29), hyperproliferation under long-term chronic inflammation status is a critical condition in carcinogenesis and inhibition of uncontrolled cell proliferation is important for the prevention of carcinoma development. Moreover, they suggested that inhibition of both cell proliferation and inflammatory activity is crucial for the development of an efficient strategy to prevent colitis associated carcinogenesis. Inositol hexaphosphate is one of such agents that exhibit the immunomodulatory action and inhibit cell proliferation. Numerous reports indicate that anti-proliferative effect of IP6 is associated with cell growth inhibition and cell cycle progression halt, modulation of cell cycle regulators expression and cyclin-dependent kinases activation. Schröterová et al. (26) underline a more universal nature of IP6 since it has shown a similar inhibitory effect in many cell types including human colon cancer cell lines HT-29, SW-480, SW-620, breast cancer cell lines MCF-7 and MDA-MB231, prostate carcionoma cell lines PC-3, TRAMP-C1 and DU145.

El-Sherbiny et al. (21) found that in MCF-7 and MDA-MB 231 and HT-29 cells IP6 controls the progression of cells through the cycle by decreasing S-phase and arresting cells in the G0/G1-phase. Moreover, they observed a significant decrease in the expression of proliferation markers Ki-67 and PCNA in IP6-treated cells. The results of Singh et al. (22) indicated a significant dose- as well as time-dependent growth inhibition in IP6-treated prostate carcinoma DU145 cells, which was associated with an increase in G1 arrest. IP6 strongly enhanced the expression of cyclin-dependent kinase inhibitors (CDKIs), Cip1/P21 and Kip1/P27, and inhibited kinase activities associated with CDK 2, 4 and 6, and cyclin E and D1. Similarly, the growth inhibitory effect of IP6 and associated mechanisms have been investigated in human prostate carcinoma LNCaP cells. IP6 treatment of cells resulted in a strong growth inhibition and an increase in G1 cell population. IP6 caused an increase in CDKIs (P21 and P27) levels, together with a decrease in cyclin-dependent kinase (CDK4) and cyclin D1 protein levels (30). The immunohistochemical studies by Gu et al. (31) showed significant reduction of the expression of cyclin D1 and proliferating cell nuclear antigen by IP6 in prostate PC-3 cells. Vucenik et al. (23) hypothesized that IP6 modulates cell cycle by interacting with cytoplasmic signaling molecules based on the observation of blockage of proliferation of MCF-7 cells through an increase in the expression of anti-proliferative PKCδ. Furthermore, an *in vitro* administration of IP6 at doses of 0.5, 1.0, and 5.0 mM for 24 and 72 h to human pancreatic adenocarcinoma cells (MIA-PACA and PANC1) significantly reduced their proliferation (24). The cDNA microarray analysis demonstrated more than 2-fold down-expression of genes encoding cyclin H and PCNA in human leukaemic K562 cells treated with 5 mM IP6 for 24 h (32).

Several studies have also evaluated the role of IP6 in colon cancer cells proliferation. Tian et al.

(25) proved a potent inhibitory effect of IP6 on growth of HT-29 cells by modulating the expression of key cell cycle regulators. According to the authors, this compound reduced proliferation rate of cells and PCNA expression together with increasing the expression of P21 protein. Likewise, the results of Schröterová et al. (26) research indicated that IP6 at doses of 0.2–5 mM markedly inhibited DNA synthesis in cell lines HT-29, SW-480 and SW-620 derived from colorectal carcinoma. Our previous experiments, based on cellular DNA content determination, have also shown that IP6 at 5 mM inhibited the growth of colon cancer HT-29 and Caco-2 cells (33). Moreover, it stimulated P21 expression at the mRNA level in HT-29 cells, with the highest increase in P21 mRNA occurring at 24 h (34).

Currently, we investigated the influence of IP6 at a concentration of 1 and 2.5 mM on the expression of cyclin D1 and histone H3 at the transcription level in proliferating Caco-2 cells under inflammatory conditions. The results indicated that IP6 altered the transcriptional activity of CCND1 and H3 genes in time-dependent manner. We observed that 6 h incubation of Caco-2 with IP6 enhanced the level of H3 mRNA stimulated by IL-1β. This was inversely correlated with IP6 concentration. The longer treatment times with IP6 (12 h), independently of its concentration caused significant down-regulation in the expression of both CCND1 and histone H3 genes.

These results confirm previously published by us and others data that IP6, a dietary agent, possesses anti-proliferative efficacy against many cancer cell types including colon cancer cells. Based on these studies it may be concluded that the ability of IP6 to inhibit colon cancer cells proliferation may be mediated through down-regulation of genes encoding cyclin D1 and histone H3 at the mRNA level. Suppressive effect of IP6 on the CCND1 and histone H3 expression may be associated with cell arrest in G1-phase, decreasing the amount of cells in S-phase of the cell cycle and DNA content.

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