

INDUCTION OF THE EXPRESSION OF GENES ENCODING TGF- β ISOFORMS AND THEIR RECEPTORS BY INOSITOL HEXAPHOSPHATE IN HUMAN COLON CANCER CELLS

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Abstract: Transforming growth factors- β (TGF- β) are multifunctional cytokines involved in the regulation of cell development, differentiation, survival and apoptosis. They are also potent anticancer agents that inhibit uncontrolled proliferation of cells. Incorrect TGF- β regulation has been implicated in the pathogenesis of many diseases including inflammation and cancer. In humans, the TGF- β family consists of three members (TGF- β 1, 2, 3) that show high similarity and homology. TGF- β s exert biological activities on various cell types including neoplastic cells *via* their specific receptors. Inositol hexaphosphate (phytic acid, IP6), a phytochemical has been reported to possess various health benefits. The aim of this study was to examine the effect of IP6 on the expression of genes encoding TGF- β 1, TGF- β 2, TGF- β 3 isoforms and their receptors T β RI, T β RII, T β RIII in human colorectal cancer cell line Caco-2. The cells were treated with 0.5, 1 and 2.5 mM IP6 for 3, 6 and 12 h. The untreated Caco-2 cells were used as the control. Quantification of genes expression was performed by real time QRT-PCR technique with a SYBR Green I chemistry. The experimental data revealed that the TGF- β 1 mRNA was the predominant isoform in Caco-2 cells and that IP6 enhanced transcriptional activity of genes of all three TGF- β isoforms and their receptors T β RI, T β RII, T β RIII in these cells. At concentrations up to 1 mM, IP6 over-expressed the genes in 6 h lasting cultures, and its higher dose (2.5 mM) caused successively increasing transcript level of TGF- β isoforms and receptors with the duration of experiment up to 12 h. The findings of this study indicate that one of anti-cancer abilities of IP6 can be realized by enhancing the gene expression of TGF- β isoforms and their receptors at the transcriptional level.

Keywords: inositol hexaphosphate, TGF- β , colon cancer, real time RT-QPCR

Transforming growth factors- β (TGF- β) are multifunctional cytokines involved in the regulation of a variety of biological processes, including cell development, differentiation, survival and apoptosis (1, 2). In humans, the TGF- β family consists of three members (TGF- β 1, 2, 3) that show high similarity and homology (3), but are encoded by different genes (4). TGF- β s exert their activities on various cells through specific receptor signaling system (T β R) (5). The TGF- β either binds to T β RIII which presents ligand to T β RII or it binds to T β RII directly. After TGF- β is bound to a heterodimeric complex of RI and RII, RI is transphosphorylated by RII. The activated RI phosphorylates selected Smad proteins. Activated Smad complexes translocate into the nucleus and regulate the transcription of a large number of TGF- β -responsive genes (6). Impaired TGF- β regulation has been implicated in

the pathogenesis of many diseases including inflammation and cancer (7). As a tumor suppressor, TGF- β has influence on proliferation, replication potential and apoptosis, and on the other hand, as a tumor promoter, it plays a crucial role in angiogenesis, migration and invasion (4). Clinical studies have shown that the levels of TGF- β are increased in colon cancer and correlate with a poor prognostic outcome (8, 9).

In recent years, there has been a growing interest in functional foods which, besides nutrient components, contain foodstuffs that improve overall health and reduce the risk of disease. One of the promising dietary phytochemicals with chemopreventive and chemotherapeutic potential is inositol hexaphosphate (phytic acid, IP6) of which large amounts have been identified in legumes, cereals, oilseeds and nuts (10, 11). It selectively inhibits can-

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cer cells without affecting the normal cells and acts synergistically with standard therapeutics (12). After cellular uptake IP6 enters inositol phosphate pool and controls a variety of cellular activities by affecting signal transduction pathways and modulating genes expression. It has been reported that IP6 inhibited proliferation, cell cycle progression, metastasis and invasion, angiogenesis and induced cell differentiation and apoptosis (13–25).

The aim of this study was to examine the effect of IP6 on the expression of genes encoding TGF- β 1, TGF- β 2, TGF- β 3 isoforms and their receptors T β RI, T β RII, T β RIII in human colorectal cancer cell line Caco-2.

EXPERIMENTAL

Cell culture

Human colon adenocarcinoma cell line Caco-2 was obtained from American Type Culture Collection. Cells were grown in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 100 U/mL penicillin (Sigma Aldrich), 100 μ g/mL streptomycin (Sigma Aldrich) and 10 mM HEPES (Gibco-BRL) at 37°C and 5% CO₂. Cells were plated 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. Afterwards cells were treated with 0.5, 1 and 2.5 mM IP6 as dipotassium salt (pH 7.4) (Sigma Aldrich) for 3, 6 and 12 h. The IP6-untreated Caco-2 cells were used as the control.

RNA extraction

Total RNA was isolated from both control and IP6-treated cells by applying a commercially available TRIZOL[®] reagent (Invitrogen) according to the producer's protocol. The quantity of RNA was evaluated by spectrophotometric measurement using the GeneQuant pro (Amersham Biosciences).

Real-time QRT-PCR assay

Transcriptional activity of examined genes was estimated on the basis of the copy number of mRNA related to 1 μ g of total RNA by the use of real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) technique with a SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit; QIAGEN) (23). The analysis was carried out using an Opticon DNA Engine Continuous Fluorescence detector (MJ Research). QRT-PCR assay was performed in triplicate for each sample.

Oligonucleotide primers specific for TGF- β 1, TGF- β 2, TGF- β 3, T β RI, T β RII and T β RIII were described previously (26, 27). Finally, specificity of RT-PCR reaction was confirmed by determining the characteristic temperature of melting for each amplicon and by 6% polyacrylamide gel (PAA) electrophoresis of RT-PCR products with their visualization using silver staining.

Statistical analysis

Statistical analysis was performed with the use of Statistica PL 9.0 software. The results of copy numbers per 1 μ g of total RNA were presented as the means \pm SD. The one-way ANOVA followed by *post-hoc* Tukey's test were applied to assess differences in the expression of examined genes between Caco-2 cell treated with IP6 and control cells. Significance level was assumed for $p < 0.05$.

RESULTS

The transcriptional activity of genes encoding TGF- β isoforms and their receptors was detected both in the control cells and the cells treated with different doses of IP6 for 3, 6 and 12 h. The experimental data revealed that the TGF- β 1 mRNA was the predominant isoform in human Caco-2 cells, as indicated by $2.61 \times 10^5 \pm 0.19$ copy numbers, and it showed significantly higher expression than others ($p < 0.001$, Tukey test). Moreover, TGF- β 2 ($0.8 \times 10^3 \pm 0.6$) and TGF- β 3 ($1.36 \times 10^3 \pm 1.00$) mRNAs were detected at the comparable level ($p = 0.9998$). There were observed no significant differences in T β RI ($4.96 \times 10^4 \pm 0.42$), T β RII ($4.79 \times 10^4 \pm 0.52$) and T β RIII ($2.16 \times 10^4 \pm 1.98$) genes expression ($p = 0.1792$; ANOVA) in Caco-2 cultures.

Furthermore, IP6 enhanced transcription of genes of all three TGF- β isoforms and their receptors in colon cancer cells in a dose and time-dependent manner. In cells exposed to IP6 at the concentrations up to 1 mM for 6 h an increase in TGF- β 1 mRNA as compared to control was detected ($p < 0.05$). After 12 h, these cultures manifested a decrease in TGF- β 1 transcripts, but the levels were significantly higher than in IP6 untreated cells ($p < 0.05$). IP6 at the highest dose (2.5 mM) evoked gradual up-regulation of TGF- β 1 expression for 3, 6 and 12 h, however, significant differences between control and IP6-treated culture were observed after 6 ($p = 0.029$) and 12 h ($p = 0.005$) stimulation (Fig. 1A). The transcription of TGF- β 2 gene was up-regulated in Caco-2 exposed to 0.5 mM ($p = 0.019$) and 1 mM IP6 ($p = 0.009$) for 3 h in comparison with untreated cells, and the copy numbers of this isoform were

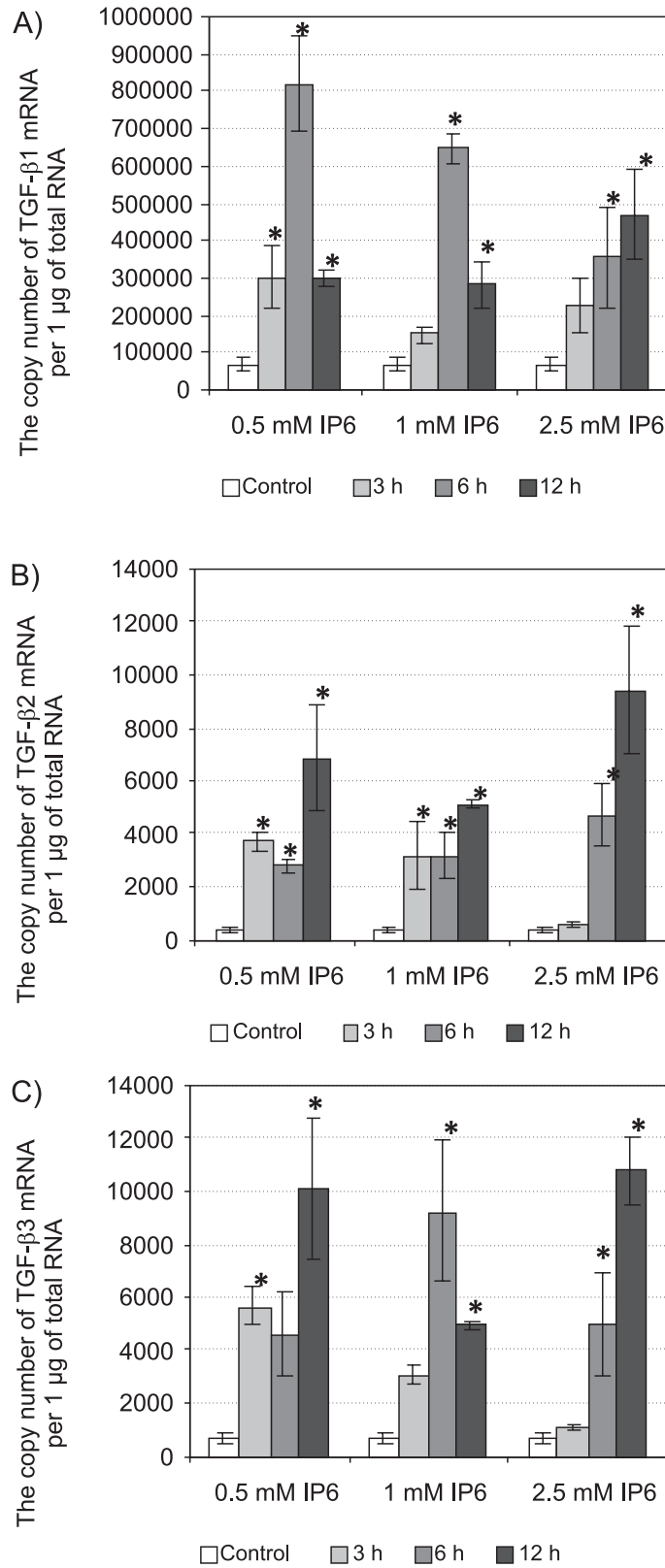


Figure 1. Comparison of (A) TGF- β 1, (B) TGF- β 2, and (C) TGF- β 3 gene expression in Caco-2 cells treated with 0.5, 1, 2.5 mM IP6 for 3, 6 and 12 h (the mean \pm SD; *p < 0.05 vs. control)

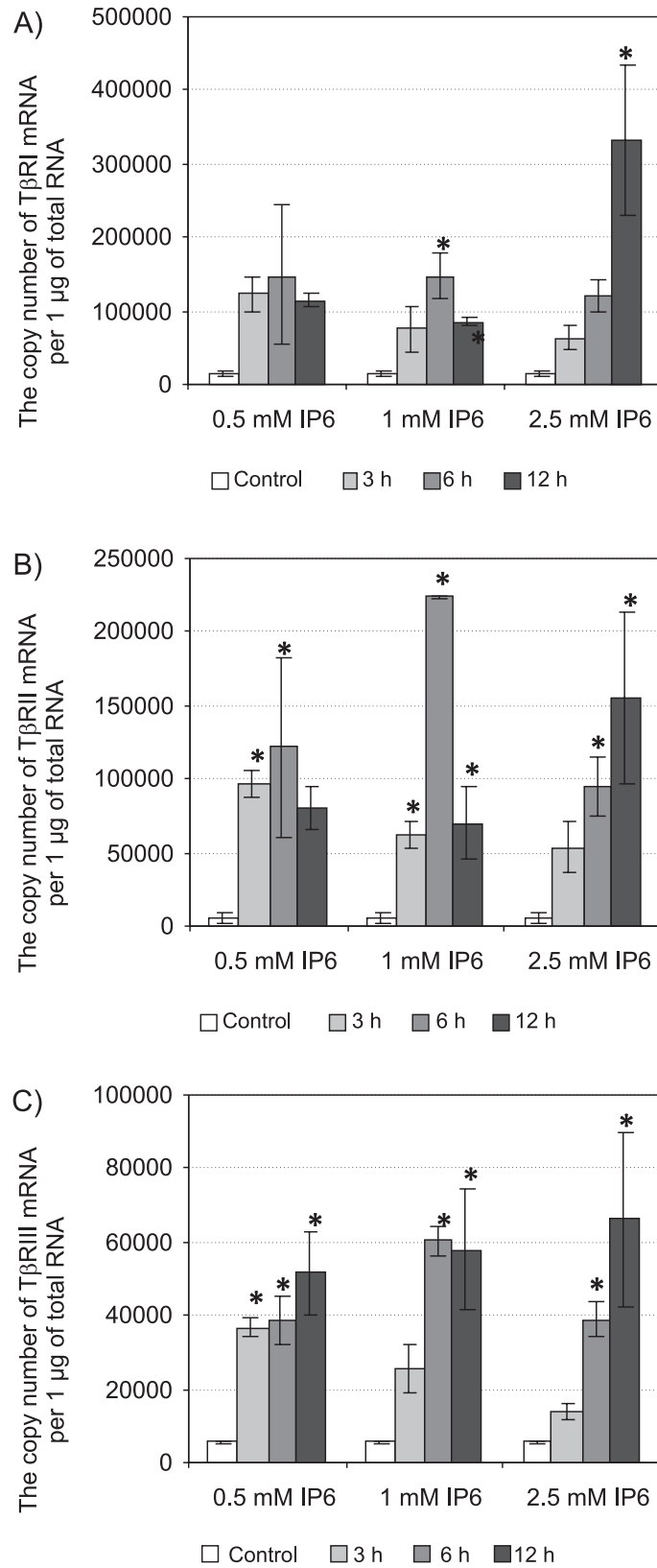


Figure 2. Comparison of (A) TβRI, (B) TβRII, and (C) TβRIII gene expression in Caco-2 cells treated with 0.5, 1, 2.5 mM IP6 for 3, 6 and 12 h (the mean \pm SD; * $p < 0.05$ vs. control)

maintained at the similar levels up to 6 h. Significantly higher quantities of TGF- β 2 mRNA were determined in cultures stimulated with IP6 at both 0.5 mM and 1 mM concentration for 12 h than in control ($p < 0.001$). The treatment of cells with 2.5 mM IP6 for 6 h ($p = 0.048$) and 12 h ($p < 0.001$) increased TGF- β 2 transcript level as compared to the control (Fig. 1B). As to TGF- β 3, IP6 of 0.5 mM induced significantly higher expression of this isoform in Caco-2 cells after 3 h ($p = 0.021$) and 12 h ($p < 0.001$). Treatment of cells with 1 mM IP6 resulted in increased expression of TGF- β 3 gene compared to the control ($p < 0.05$) with the maximum level seen after 6 h stimulation. The markedly higher level of TGF- β 3 transcript was produced in response to 2.5 mM IP6 than in control cells after both 6 h ($p = 0.046$) and 12 h ($p < 0.001$) (Fig. 1C).

When Caco-2 cells were treated with IP6 of 0.5 mM, an increase in T β RI expression after 3, 6 and 12 h, however, not statistically significant ($p > 0.05$) could be detected. Cells exposed to 1 mM IP6 produced significantly higher amount of T β RI transcript compared to control for 6 h ($p = 0.001$) and 12 h ($p = 0.034$). IP6 2.5 mM overexpressed T β RI mRNA only after 12 h stimulation ($p = 0.022$) (Fig. 2A). Transcriptional activity of T β RII gene increased in cells incubated with IP6 at both 0.5 mM and 1 mM for 3 and 6 h and was reduced with duration of experiment up to 12 h ($p < 0.05$). In a time course experiment, up-expression of T β RII mRNA was observed in cells stimulated with 2.5 mM IP6 as compared to control (Fig. 2B). Comparative analysis of T β RIII mRNA copies per 1 mg of total RNA revealed higher quantities in cell cultures incubated with IP6 at all applied doses for 3, 6 and 12 h than in control cells (Fig. 2C).

DISCUSSION and CONCLUSION

To determine the effect of IP6 on the expression of TGF- β isoforms and their receptors in colon cancer cells, we first examined their basal expression pattern. Three TGF- β isoforms are present in mammalian cells and expressed in a tissue-specific regulated manner. Of these, TGF- β 1 is most frequently up-regulated isoform in tumor cells (4, 5, 28). The TGF- β receptors (T β RI, T β RII, T β RIII) have been identified in most cells including tumor cells (6). Where expressed, T β RIII is the most abundant TGF- β receptor (4), as well as T β RII and T β RI show a widespread pattern of expression (5). Eckmann et al. (29) assessed the expression of TGF- β 1 in human colon epithelial cell lines T84, Caco-2, SW620 and HT29 by using RT-PCR. The cell lines

constitutively expressed TGF- β 1 mRNA in the absence of added stimuli. Barnard et al. (30) revealed co-localized TGF- β 1, - β 2 and - β 3 mRNAs in mouse normal colon epithelium. The present study demonstrated transcriptional activity of all TGF- β and receptor genes in Caco-2 cells and confirmed that the TGF- β 1 mRNA was the predominant isoform. However, the expression of genes encoding TGF- β receptors was at the similar level in this cell line.

In the gastrointestinal tract, TGF- β can either promote or suppress cancer formation (31). It is a potent anticancer agent that inhibits the uncontrolled proliferation of cells (32) and prevents early transition from hyperplasia to dysplasia (9). At the late stage of tumor development, TGF- β signaling is a crucial pathway for tumor growth, invasion and metastasis (33). According to Hong et al. (33) restoration or augmentation of TGF- β signal transduction can be a good strategy for cancer prevention and treatment. Based on tumor suppressor effect of TGF- β , attempts to increase its activity could be used as a therapy for early-stage disease (4). On the other hand, many human cancers become resistant to the antiproliferative effects of TGF- β due to mutation of TGF- β receptor genes or their attenuated expression. In these cases, increasing expression of the receptors may be a reasonable therapeutic target (4). Therefore, compounds which enhance expression of receptors could potentially be used in therapy of colon cancer that commonly displays decreased expression levels of T β RII (4, 33).

Recently, the use of naturally occurring substances like resveratrol, genistein or phytic acid is considered as a priority to chemoprevention and cancer therapy. *In vitro* and *in vivo* studies have demonstrated the influence of these nutraceuticals on TGF- β expression. The experiment by Schneider and co-workers (34) showed that oral administration of resveratrol stimulated by 1.7-fold the expression of TGF- β gene in the small intestinal mucosa of mice. In another study, Yu et al. (35) revealed increased level of TGF- β 1 mRNA in mouse colon cancer MC-26 cells treated with genistein. The authors postulated that genistein and TGF- β 1 have similar inhibitory effects on cell growth, including regulating cell proliferation, apoptosis, differentiation, and they speculated that the actions of one might involve or even require the other (35). The published data demonstrated that IP6 also has similar inhibitory properties to TGF- β . Both induce G1 arrest in cell cycle progression and inhibit cell growth and proliferation (4, 7, 18, 25).

In this study we have analyzed the effect of IP6 on the expression of genes encoding TGF- β 1, TGF- β 2, TGF- β 3 isoforms and their receptors T β RI, T β RII, T β RIII in human colorectal cancer cell line Caco-2. Ours is the first study to demonstrate the influence of IP6 simultaneously on the expression of three TGF- β isoforms and their receptors in colon cancer cells. Previously, Marks et al. (36) evaluated modulation in the expression of TGF- β 2 in colon carcinogenesis. The administration of IP6 to mice promoted an increase in TGF- β 2 expression, though not in a significant manner, as compared with the control group. Microarray experiment of Bozsik et al. (23) revealed that treatment of K-562 human leukemia cells with 750 mM IP6 for 60 min resulted in modulation of expression of genes encoding some proteins involved in TGF- β signaling pathway. The results of our study revealed that IP6 stimulated transcriptional activity of genes of all three TGF- β isoforms and their receptors T β RI, T β RII, T β RIII in Caco-2 cells. At concentrations up to 1 mM, IP6 over-expressed the genes in 6 h lasting cultures, and its higher dose (2.5 mM) caused gradually increasing transcript level of TGF- β isoforms and receptors with the duration of experiment up to 12 h.

In conclusion, the findings of this study show that IP6 promoted the expression of TGF- β isoforms and those of their receptors in colon cancer cells at the transcriptional level in a way dependent on its concentration and time of interaction. Thus, inositol hexaphosphate can inhibit tumor development in early stage by enhancing the expression of TGF- β capable of suppressing tumor growth and by increasing of TGF- β receptors expression to prevent cancer progression.

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