# INHIBITORY EFFECT OF INOSITOL HEXAPHOSPHATE ON METALLOPROTEINASES TRANSCRIPTION IN COLON CANCER CELLS STIMULATED WITH PHORBOL-12-MYRISTATE 13-ACETATE

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**Abstract:** Inositol hexaphosphate (IP6) is a naturally occurring phytochemical, found in abundance in cereals, legumes and other high-fiber-content diets. IP6 has shown promising efficacy against a wide range of cancers. Its anti-cancer activity involves anti-proliferative, pro-apoptotic and anti-metastatic effects. Both matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), are implicated in tumor growth, metastasis, and angiogenesis. Phorbol-12-myristate 13-acetate (PMA) is a well-known inflammatory stimulator and tumor promoter that activates PKC and increases the invasiveness of various types of cancer cells by activating MMPs. The aim of the present study was to examine the influence of IP6 on the expression of selected MMPs, i.e., MMP-1, -2, -3, -9, 10, -13 and their TIMP-1 and -2 in unstimulated and PMA-stimulated colon cancer cell line Caco-2. Quantification of genes expression in Caco-2 cells treated with 100 ng/mL of PMA, 2.5 mM of IP6 and both for 6 and 12 h was carried out using real time QRT-PCR technique. Stimulation of cells with PMA resulted in an up-expression of MMP-2, MMP-3, MMP-9, MMP-10, MMP-13 and TIMP-1 mRNAs and decrease in MMP-1 gene expression. The quantity of TIMP-2 transcript was reduced by PMA. A significant decrease in MMP-2, MMP-3, MMP-10, MMP-13, and TIMP-1 expression in response to IP6 was observed. IP6 down-regulated MMP-9 transcription induced by PMA and decreased the level of both MMP-2 and MMP-3 mRNAs in PMA-stimulated cells. Caco-2 treated with both PMA and IP6 showed a significant decrease in MMP-1 expression in comparison to PMA-stimulated cells. The results of this study show that PMA can modulate MMP and TIMP genes transcription in colon cancer cells Caco-2. IP6 exerts an influence of basal mRNA expression of some MMPs and their tissue inhibitors and down-regulates MMP-1, MMP-2, MMP-3 and MMP-9 in cells treated with PMA. IP6 could be an effective anti-metastatic agent that suppresses expression of MMP genes at transcription level.

**Keywords**: IP6, matrix metalloproteinases, colon cancer, phorbol-12-myristate 13-acetate, mRNA quantification

The present therapies including surgery, chemotherapy, and radiotherapy are still limited for advanced colon tumors. Therefore, a growing attention has been focused on investigating the potential of dietary components for both prevention and control of cancer through chemopreventive strategies (1). Inositol hexaphosphate (phytic acid, IP6) is a naturally occurring phytochemical, found in abundance in cereals, legumes and other high-fiber-content diets (2). In mammals, IP6 and its lower phosphorylated derivates participate in regulating vital cellular functions, particulary cell division and differentiation (3, 4). IP6 has also been reported to possess antioxidant, anti-inflammatory and immune enhancing function. The most striking effect of IP6 has been documented in cancer prevention by con-

trolling tumor growth, progression and metastasis. *In vitro* and *in vivo* studies showed that IP6 targets cancer through multiple pathways and signaling molecules (5–8). It has been found to block phosphatidylinositol-3 kinase (PI3K), activating protein-1 (AP-1), protein kinase C (PKC), and mitogen-activated protein kinases (MAPK), as well as nuclear factor κB (NFκB) (4, 9–12).

Matrix metalloproteinases have long been associated with cancer invasion and tumor progression. Their expression and activation is increased in almost all human cancers compared with normal tissue. These enzymes comprise a family of zinc-dependent endopeptidases that can be divided into several subgroups based on their structure and substrate specificity. They include collagenases (MMP-

1, MMP-8, MMP-13, MMP-18), stromelysins (MMP-3, MMP-10, MMP-11), gelatinases (MMP-2, MMP-9), matrilysins (MMP-7, MMP-26), enamelysins (MMP-18, MMP-19), metalloelastases (MMP-12), membrane-type metalloproteinases (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25) and others. Their activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) which bind covalently to the active site of the enzyme (13–16).

The expression of MMPs can be stimulated by various agents, such as inflammatory cytokines, growth factors, tumor promoters, and also by oncogenic transformation and cell-matrix and cell-cell interactions (14). Phorbol-12-myristate 13-acetate (PMA) is a well-known inflammatory stimulator and tumor promoter that activates protein kinase C (PKC) and increases the invasiveness of various types of cancer cells by activating MMPs. It controls the expression of genes by modulating the activation of transcription factors such as AP-1 and NF-κB through MAPK signaling pathways (17–19).

Since the expression of MMPs and TIMPs is highly regulated by signal transduction pathways involving PKC (18), we investigated the effect of PMA on MMP and TIMP expression and the ability of IP6 to affect PMA-influenced changes in mRNA levels of the studied genes.

## EXPERIMENTAL

### Cell culture

The Caco-2 human colon adenocarcinoma cells (ATCC) were cultured in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/mL penicillin and 100 μg/mL streptomycin (both from Sigma Aldrich) and 10 mM HEPES (Gibco BRL). They were maintained at 37°C in a 5% $CO_2$ atmosphere. For experiments, the cells were seeded at a density of $1 \times 10^6$ per 5 mL medium in 21.5 cm$^2$ culture flasks. After 3 days, the media containing serum were changed to serum-free media and the cells were cultured for 24 h. They were then stimulated with 100 ng/mL PMA (Sigma Aldrich) for 30 min. Afterwards, the cells were treated with 2.5 mM IP6 (Sigma Aldrich) for 6 and 12 h. In separate cultures, cells were treated with PMA or IP6 alone at the indicated concentrations for the indicated times. The untreated Caco-2 cells were used as the control.

### RNA extraction

Stimulated cells were subjected to the extraction of total RNA with the use of TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. The quality of the extracted RNA was evaluated by electrophoresis in 1.0% agarose gel stained with ethidium bromide. RNA concentration was determined spectrophotometrically using a GeneQuant pro (Amersham Biosciences).

### Real-time QRT-PCR

Detection of the expression of MMP and TIMP mRNAs was carried out using a QRT-PCR technique with a SYBR Green chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen) and Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research) as described previously (20). Oligonucleotide primers specific for MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-13, TIMP-1 and TIMP-2 mRNAs were designed using Primer Express 2.0 software (PE Applied Biosystems, USA) and presented elsewhere (21). QRT-PCR assay was performed in triplicate for each sample. The obtained results of mRNA copy number were recalculated per μg of total RNA.
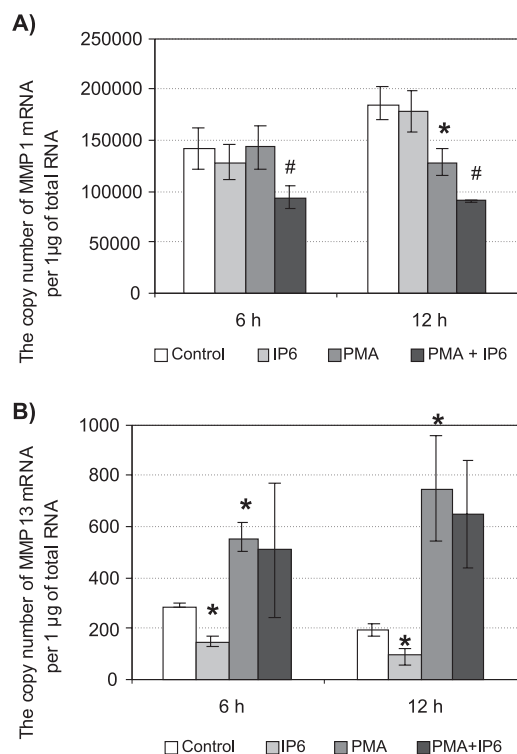


Figure 1. Evaluation of expression of genes encoding collagenases in Caco-2 cells treated with 2.5 mM IP6 and PMA for 6 and 12 h. Comparison of the transcriptional activity of **A**) MMP-1 and **B**) MMP-13 genes in Caco-2 cells (the mean ± SD; * p < 0.05 *vs.* control; # p < 0.05 *vs.* PMA-stimulated cells)
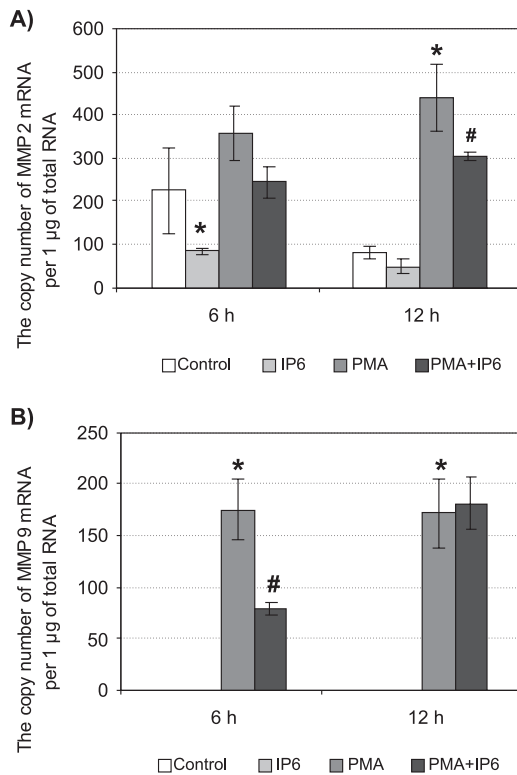
A)



B)



Figure 2. Evaluation of expression of genes encoding gelatinases in Caco-2 cells treated with 2.5 mM IP6 and PMA for 6 and 12 h. Comparison of the transcriptional activity of **A**) MMP-2 and **B**) MMP-9 genes in Caco-2 cells (the mean ± SD; * p < 0.05 *vs*. control; # p < 0.05 *vs*. PMA-stimulated cells). In Caco-2 cells we did not detect the expression of MMP-9 gene either under normal conditions or following treatment with 2.5 mM IP6 for each interaction time
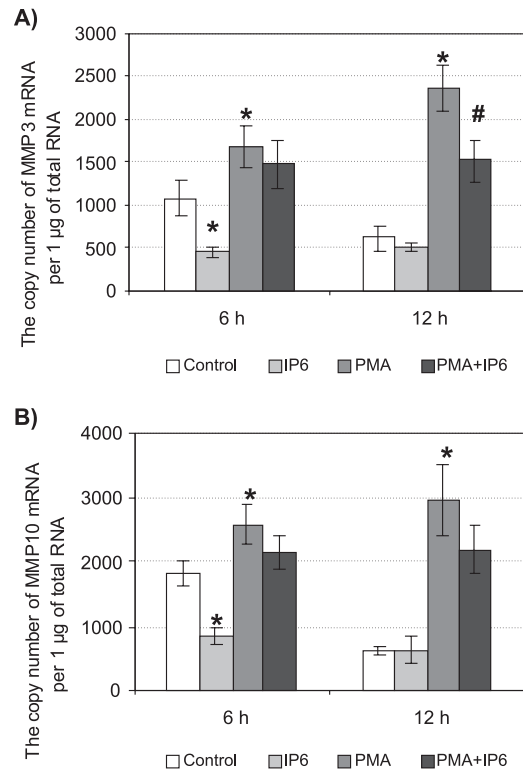
A)



B)



Figure 3. Evaluation of expression of genes encoding stromelysins in Caco-2 cells treated with 2.5 mM IP6 and PMA for 6 and 12 h. Comparison of the transcriptional activity of **A**) MMP-3 and **B**) MMP-10 genes in Caco-2 cells (the mean ± SD; * p < 0.05 *vs*. control; # p < 0.05 *vs*. PMA-stimulated cells)

**Statistical analysis**

Statistical analysis was performed using the Statistica PL 9.0 software. All the results are expressed as the means ± SD. Comparison of two data sets was performed by two-tailed unpaired t test. Comparison of more than two data sets was performed by one-way ANOVA; p < 0.05 was accepted as significant.

**RESULTS**

A comparison of the transcriptional activity level of genes encoding MMPs and TIMPs in the control cells and cultures treated with IP6 and PMA for 6 and 12 h showed significant differences (p < 0.05; ANOVA).

At first, to investigate whether PMA modulates expression of examined MMP and TIMP genes,

Caco-2 were treated with PMA at concentration of 100 ng/ml for 6–12 h. The obtained results of real time QRT-PCR assay indicated significant up-regulation of MMP-13, -9, -3, -10 and TIMP1 mRNAs in PMA-treated cells for both 6 and 12 h (p < 0.05, t-test) (Fig. 1B, 2B, 3, 4A). A longer stimulation of cells (12 h) with PMA caused an increase in MMP-2 (p < 0.0001) (Fig. 2A) and a decrease in MMP-1 expression (p = 0.0084) (Fig. 1A) in relation to control. The quantity of TIMP-2 transcript was reduced by PMA compared to unstimulated cells at both time points (p < 0.05; Fig. 4B).

Analysis of experimental data revealed that 2.5 mM IP6 had no influence on MMP-1 expression (p > 0.05), but it significantly down-regulated this gene in PMA-stimulated cells at 6–12 h (p < 0.05) (Fig. 1A). Furthermore, IP6 reduced the level of MMP-13 mRNA in 6 and 12 h cultures (p < 0.05), and has no
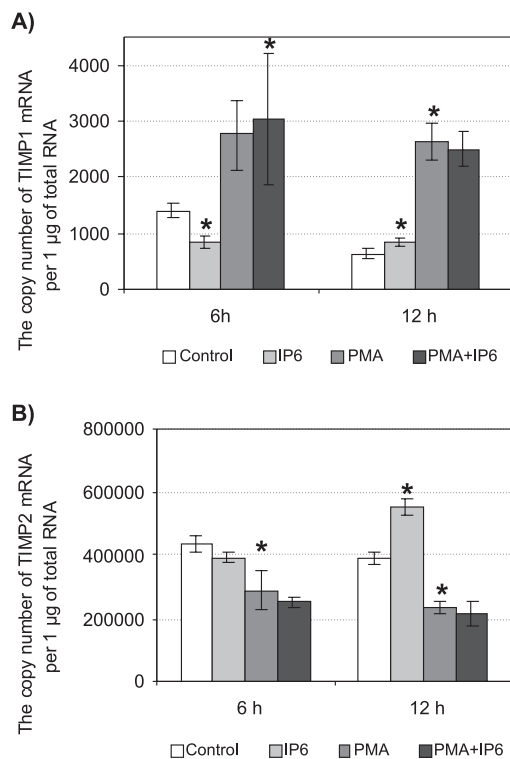
**A)**



**B)**



Figure 4. Evaluation of expression of genes encoding inhibitors of matrix metalloproteinases in Caco-2 cells treated with 2.5 mM IP6 and PMA for 6 and 12 h. Comparison of the transcriptional activity of **A**) TIMP-1 and **B**) TIMP-2 genes in Caco-2 cells (the mean ± SD; * p < 0.05 *vs*. control)

effect on this gene transcription in cells treated with PMA (Fig. 1B).

Treatment with IP6 led to decrease in the transcriptional activity of MMP-2 in comparison to control with significant difference observed at 6 h (p = 0.0487). Stimulation of Caco-2 with PMA/IP6 for 12 h resulted in a down-regulation of MMP-2 gene as compared with PMA-treated cells (p = 0.0433) (Fig. 2A). The MMP-9 gene was not expressed in cells either under normal conditions or following treatment with 2.5 mM IP6 for each of the incubation times. However, its transcription was induced by PMA in all cell cultures (6–12 h). The ability of PMA to up-regulate MMP-gene in 6 h lasting cultures was overcome by IP6 (p = 0.0051). At 12 h, the level of MMP-9 mRNA in cells treated with PMA and PMA/IP6 was quantitatively similar (p = 0.7346) (Fig. 2B).

IP6 at concentration of 2.5 mM evoked a decrease in the expression of genes encoding

stromelysins (MMP-3 and MMP-10) in comparison to unstimulated cells for 6 h (p < 0.05). Furthermore, both genes did not show any changes in transcriptional activity in cultures after 12 h incubation with IP6 (Fig. 3). A significantly lower MMP-3 mRNA level was determined following PMA and IP6 treatment of cells for 12 h than in PMA-stimulated cells (p = 0.0164) (Fig. 3A). On the contrary, no marked differences in MMP-10 gene activity were manifested by PMA-stimulated cells and those treated with PMA and IP6 for 6–12 h.

IP6 down-regulated transcriptional activity of TIMP-1 gene in Caco-2 at 6 h (p = 0.0067). The level of both TIMP-1 and TIMP-2 mRNAs in cells exposed to IP6 for 12 h was significantly higher than in untreated cells (p < 0.05). Furthermore, IP6 had no influence on the expression of both genes encoding inhibitors in Caco-2 cells stimulated by PMA (Fig. 4).

## DISCUSSION AND CONCLUSION

This study was conducted to evaluate the efficacy of inositol hexaphosphate in regulating PMA-induced expression of MMP and TIMP mRNAs in human colon cell line Caco-2. The expression level of MMPs in unstimulated cells in culture and in intact tissues *in vivo* is low. Transcription and synthesis of MMP is known to be regulated by cytokines and growth factors implicated in various pathological processes. Caco-2 cell has previously been shown to express MMP and TIMP genes in response to IL-1b (21). Currently we used PMA, a high affinity ligand for PKC, as a model agent to stimulate the expression of MMPs in this cell line. PKC activation is suggested to be prerequisite for the stimulation of MMP genes expression (22).

Many stimulators transmit signal to the nucleus primarily *via* MAPK-cascades. In eukaryotic cell three major MAPK families: c-Jun NH2-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) have been identified. The JNKs and p38 kinases are activated in response to inflammatory cytokines, osmotic stress and apoptotic signals, while the ERKs respond to growth factors and phorbol esters. Protein kinase C (PKC) has been shown to activate ERK1,2 signaling pathway (23, 24). An extracellular stimulator could activate synchronously several members of MAPK families (25). It may lead to activation of transcription factors, such AP-1 and NF-κB and then, transcription of MMPs (24). The mutation of the AP-1 and NF-κB binding sites decreased the response to PMA (23). The results of the present study revealed the

ability of PMA to up-express of MMP-3, MMP-9, MMP-10, MMP-13 and TIMP-1 mRNAs at 6 and 12 h in Caco-2. Longer (12 h) stimulation of cells with PMA evoked an increase in MMP-2 and a decrease in MMP-1 genes expression. The quantity of TIMP-2 transcript was reduced by PMA compared to control cells at both 6 and 12 h.

Clinical studies have demonstrated enhanced expression of MMPs and TIMPs during formation, development and progression of human cancers, and especially that of colon. Among MMPs, the gelatinases (MMP-2 and MMP-9) have been most consistently detected in malignant tissues and associated with tumor aggressiveness, metastatic potential and a poor prognosis. Most cases of colorectal carcinogenesis are characterized by enhanced expression of MMP-7, -13, -3 (26–28). During the last several years, an increasing number of reports have documented the presence of interstitial collagenases in aggressive tumors, suggesting a definite correlation between this subfamily of MMPs and tumor prognosis (29). Moreover, TIMP-1 expression level was found higher in cancer tissue than in adjacent normal mucosa (28).

The crucial goal of chemoprevention by natural dietary compounds is the reduction of cancer incidence by intervening in pathways which promote growth and metastasis of colon cancer (1). The mechanisms of their action could be associated with the modulation of genes expression involved in the regulation of these processes. Inositol hexaphosphate shows this characteristics and influences gene expression at the transcriptional level. We previously observed the alteration of MMP and TIMP genes expression by IP6 in colorectal cancer cell line Caco-2 (20). IP6 was also an efficient down-regulator of MMP-1, MMP-9 and TIMP-2 genes transcription stimulated by IL-1β in 6 h lasting culture. After 12 h, IL-1β-induced MMP-2 mRNA expression was significantly reduced by IP6 (21). The results of the present study revealed a significant decrease in MMP-2, MMP-3, MMP-10, MMP-13 and TIMP-1 expression in response to 2.5 mM IP6. Moreover, IP6 down-regulated MMP-9 transcription induced by PMA in 6 h lasting culture and decreased the level of both MMP-2 and MMP-3 mRNAs in PMA-stimulated cells for 12 h. Caco-2 treated with both PMA and IP6 for 6–12 h showed a significant decrease in MMP-1 expression in comparison to PMA-stimulated cells.

The inhibitory effects of naturally occurring compounds on the PMA-enhanced expression of MMPs were also examined by others in *in vitro* experiments. Woo et al. (30) found that resveratrol, a phytoalexin present in grapes, significantly inhibited the PMA-induced increase in MMP-9 expression and activity in Caski cells, a human cervical cancer cell line. These effects of resveratrol were dose dependent and correlated with the suppression of MMP-9 mRNA expression levels. A decrease of MMP-2 and MMP-9 by the nutrition mixture containing green tea extract (tea polyphenols) has been observed in both lung cancer cells A-549 and malignant mesothelioma cells MSTO-211H treated with PMA (31). The results of Park et al. (32) study indicate that kalopanaxsaponin A – oleanane triterpene saponin found in *Kalopanax pictus* Nakai (Araliaceae), alleviated metastasis by reducing MMP-9 expression and secretion as well as TIMP-1 expression in PMA-treated human breast cancer cells MCF-7. Hwang and co-workers (33, 34) investigated the inhibitory effects of bergamottin from *Citrus paradisi* (grapefruit) (33) and piperine, a major component of black pepper (34), on tumor invasion and migration and the possible mechanisms involved using human fibrosarcoma HT-1080 cells. Both agents suppressed PMA-enhanced expression of MMP-9 protein, mRNA and transcription activity levels through suppression of NF-κB and AP-1. They also reduced PMA-enhanced MMP-2 expression through suppression of membrane-type 1 MMP, without changing the level of TIMP-1 and TIMP-2.

In conclusion, the results of this study show that PMA, protein kinase C activator, can modulate MMP and TIMP genes transcription in colon cancer cells Caco-2. Two and a half mM IP6 exerts an influence on basal mRNA expression of some MMPs and their tissue inhibitors and down-regulates MMP-1, MMP-2, MMP-3 and MMP-9 in cells treated with PMA. Taken together, these findings indicate that IP6 could be an effective anti-metastatic agent that suppresses expression of MMP genes at transcription level.

## Acknowledgment

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