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DOWN-REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION BY INOSITOL HEXAPHOSPHATE IN HUMAN COLON CANCER CELLS *

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Abstract: Inflammatory bowel disease (IBD) is chronic inflammatory condition associated with increased risk of developing colorectal cancer. A number of mediators of inflammation, such as pro-inflammatory cytokines, prostaglandins and nitric oxide have been involved in carcinogenesis, especially in the promotion and progression stages. NO is synthesized from L-arginine by constitutively expressed endothelial and neuronal nitric oxide synthases (eNOS and nNOS, respectively) and an inducible NOS (iNOS) isoform expressed under inflammatory conditions. A selective inhibitors of iNOS could be, therefore, considered to be good candidates as chemopreventive agents against colon cancer. In this study, the effect of inositol hexaphosphate (IP6), dietary phytochemical, on the mRNA expression of iNOS stimulated with bacterial lipopolysaccharides (*Escherichia coli* and *Salmonella typhimurium*) and IL-1β in intestinal cells Caco-2 for 6 and 12 h was investigated. A transcription level of iNOS with the use real time QRT-PCR technique was determined in cells treated with 1 and 2.5 mM IP6. Stimulation of Caco-2 with pro-inflammatory factors (LPS and IL-1β) resulted in an up-expression of iNOS mRNA at 6 and 12 h. Cells exposed to IP6 only revealed significant reduction in iNOS gene transcription after 12 h. A decrease in iNOS transcription by IP6 following the gene induction by proinflammatory agents in 6 and 12 h lasting cultures was also determined. The findings of this study suggest that one of the anticancer and anti-inflammatory abilities of IP6 can be realized by suppressing the expression of gene encoding inducible nitric oxide synthase isoform at the transcriptional level.

Keywords: IP6, inducible nitric oxide synthase, inflammation, colon cancer

Inflammatory bowel disease (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), is defined as a chronic inflammation of the gastrointestinal tract, characterized by intestinal mucosa infiltration of monocytes and neutrophils, prolonged and exaggerated activity of pro-inflammatory cytokines and frequently by genetic predisposition and environmental contributory factors (1). The association of cancer with chronic inflammation in cancer patients has been recognized for some time and become the focus of experimental tumor systems (2, 3). Cancers associated with chronic inflammation include colorectal cancer (CRC), the fourth most common cause of death from cancer worldwide. The risk of developing CRC increases with longer duration, greater anatomic extend and severity of colitis (4, 5). Several inflammation-associated genes show up-expression in inflamed mucosa and remain elevated in colonic neoplasms (6). One of the genes, which are related both to inflammation and cancer encodes inducible nitric oxide synthase.

Nitric oxide synthase family catalyzes the synthesis of nitric oxide (NO) in the reaction of oxidative deamination of L-arginine. It consist of two constitutively expressed isoforms: endothelial (eNOS) and neural nitric oxide synthase (nNOS), and an inducible NOS (iNOS) isoform expressed under inflammatory conditions (7). iNOS is regulated at the transcriptional level in response to cytokines (IFN-γ, TNF-α, IL-1β and IL-6) and bacterial lipopolysaccharides (LPS), and does not require calcium for its activity (8). As opposed to constitutive isoforms, iNOS produces large quantities of nitric oxide (9).

NO synthesized by constitutive isoforms of NOS has vital functions in various physiological processes, such as regulation of blood pressure, smooth muscle relaxation, neurotransmission, gene

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transcription, mRNA translation and protein post-translational modifications (10). However, its overload, resulting from iNOS activity, can be detrimental and may damage healthy tissues, leading to up-regulation of inflammatory response. NO can exert cytotoxic effect *via* contribution to membrane lipid peroxidation, increased membrane permeability as well as DNA damage and suppression of DNA repair enzymes (11). NO plays a role in carcinogenesis, especially in the promotion and progression stages by stimulating cell proliferation, regulating angiogenesis and migration of tumor cells (12).

Overexpression of iNOS has been reported in chronic inflammatory diseases as well as in various types of cancers (13). Some clinical studies have shown that in patients with active UC and CD there is a substantial increase of iNOS activity (14). Up-regulation of the iNOS gene at both mRNA and protein level has also been revealed in the colorectal cancer as compared with normal mucosa (15). Therefore, selective inhibitors of iNOS could be considered to be good candidates as chemopreventive agents against IBDs and colon cancer.

In recent years, it has been shown that some plant-derived compounds demonstrate anti-inflammatory activity by controlling the levels of various inflammatory cytokines or mediators, including IL-1, IL-6, IL-10, TNF-α, NF-κB, NO, iNOS and COX-2 (16). Inositol hexaphosphate (IP6) is a natural fiber-associated dietary component, especially abundant in cereals, legumes and wheat bran. It has been involved in a variety of cellular functions such as signal transduction, regulation of cell proliferation, and differentiation (17). Its anticancer efficacy has been proved both *in vivo* and *in vitro* on various cancer cells lines. IP6 is also known for its antioxidant action, resulting from inhibition of free radical production and lipid peroxidation in the colon (18). Due to its limited toxicity on normal cells and noticeable lack of side effects IP6 is worth of considering as a preventic agent for inflammatory bowel diseases and colorectal cancer (19).

In this study the effect of inositol hexaphosphate on the mRNA expression of iNOS stimulated with bacterial lipopolysaccharides (*Escherichia coli* and *Salmonella typhimurium*) and IL-1β in intestinal cells Caco-2 was investigated.

EXPERIMENTAL

Cell culture

The human intestinal epithelial cells of Caco-2 line (DSMZ, Braunschweig, Germany) were grown 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). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were seeded into six-well plates (Nunc International) at a density of 4.5×10^5 per well 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 stimulated with 100 (μg/mL LPS (Escherichia coli serotype 055:B5, Salmonella enterica serotype typhimurium; both from Sigma Aldrich), or 1 ng/mL IL-1β (Sigma Aldrich) for 30 min. Subsequently, cells were treated with 1 and 2.5 mM IP6 as dipotassium salt (pH 7.4) (Sigma Aldrich) for 6 and 12 h. In separate cultures, cells were incubated with LPS or IL-1β under the same conditions. 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) following the manufacturer's instruction. The quality and quantity of the extracts was determined spectrophotometrically using a GeneQuant pro (Amersham Biosciences). The transcription level of iNOS gene was evaluated by the use of real-time RT-QPCR technique with SYBR Green Quantitect RT-PCR Kit (Qiagen) according to the producer's specifications. The analysis was carried out using an Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research). Oligonucleotide primers specific for mRNA iNOS were designed on the basis of reference sequence (GenBank accession no. NM_000625) by Primer Express™ Version 2.0 software (PE Applied Biosystems, USA). The following primer sequences were used: 5'-CAAGCCTACCC-CTCCAGATG-3' (forward), 5'-CATCTCC-CGTCAGTTGGTAGGT-3' (reverse). 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. 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 examined 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.

Specificity of RT-PCR reaction was confirmed by determining the characteristic temperature of melting for all amplimers (Tm = 79°C). Additionally, the RT-PCR products were separated on 6% polyacrylamide gels and visualized with silver salts. Gel electrophoresis revealed the presence of single products of 161 bp size.

Statistical analysis

The results were obtained 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 data sets was performed by one-way ANOVA followed by *post-hoc* Tukey test. Significance level was assumed for $p < 0.05$.

RESULTS

The colon cancer cells Caco-2 exhibited basal expression of the gene encoding inducible nitric oxide synthase. IP6 at concentration of 1 mM had no effect on the transcriptional activity of iNOS after 6 h treatment ($p = 0.764$). The treatment of cells with higher dose of IP6 (2.5 mM) decreased the amount of mRNA in comparison to control, however, statistical analysis revealed a trend towards significance ($p = 0.07$). Cells exposed to IP6 at both concentrations showed significant reduction in iNOS gene transcription after 12 h ($p < 0.05$) (Fig. 1).

Over the period of the experiment, stimulation of cells with pro-inflammatory factors (LPS and IL-1β) resulted in an up-expression of iNOS mRNA compared to control culture ($p < 0.001$). The obtained results revealed that all the pro-inflammatory agents induced transcriptional activity of this gene at a similar level. At 6 h, above 2-fold increase in the expression of iNOS was observed and the prolongation of time to 12 h led to its about 6-fold enhancement (Fig. 2). IP6 in a dose-independent manner was able to down-regulate iNOS transcription in cells treated with pro-inflammatory agents for 6-12 h ($p < 0.05$). The longer IP6 action (12 h) affected the stronger suppression of iNOS expression (Figs. 3-5).

DISCUSSION and CONCLUSION

Inflammatory bowel diseases are characterized by inflammatory activity of intestinal mucosa. Initiation and maintenance of inflammation is, in part, a result of sustained and elevated level of NO and inducible nitric oxide synthase (14). A number of inflammatory mediators, such as cytokines, prostaglandins and NO have been involved in carcinogenesis, especially in its promotion and progression stages. NO enhances tumor cell proliferation and promotes angiogenesis, possibly by stimu-

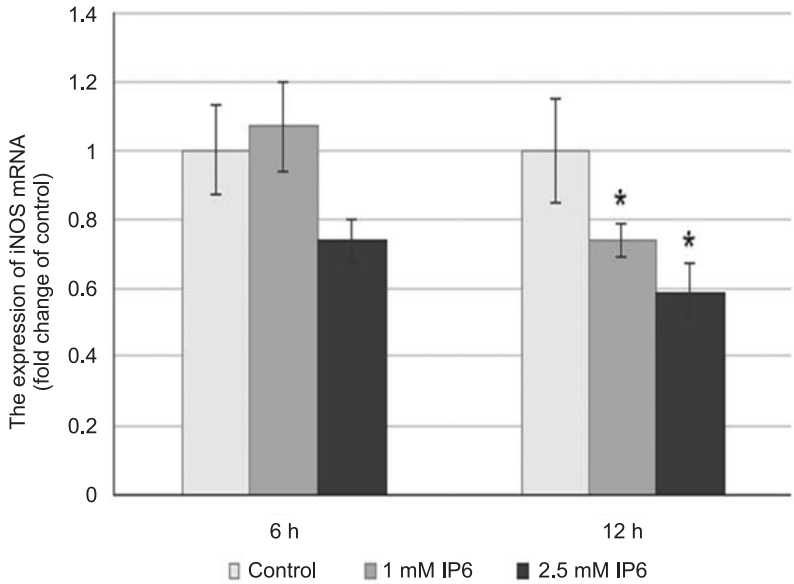

Figure 1. The effect of 1 and 2.5 mM IP6 on the expression of iNOS mRNA in Caco-2 cells. The results are presented as the mean ± SD of three separate experiments; (*$p < 0.05$ *vs.* control Caco-2 cells)

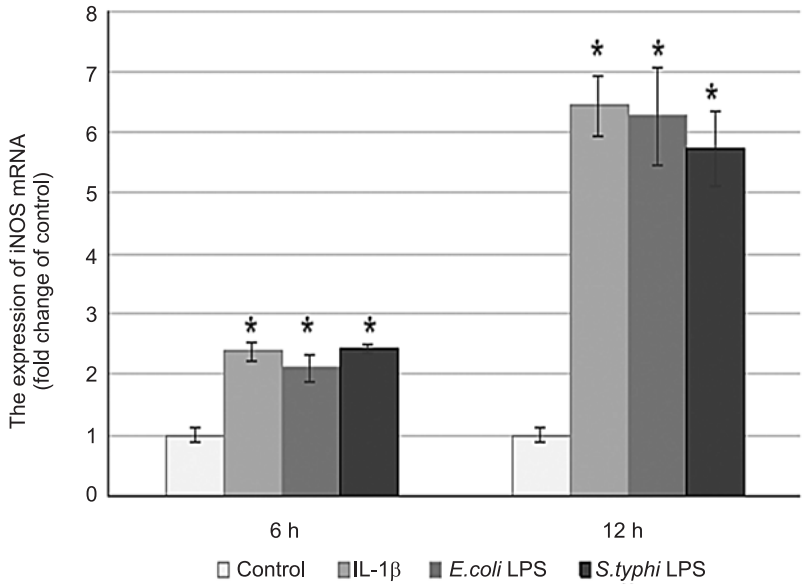

Figure 2. Changes in iNOS mRNA expression in Caco-2 cells treated with IL-1β, *E. coli* LPS and *S. typhi*. LPS for 6 and 12 h. The results are presented as the mean ± SD of three separate experiments; (*p < 0.05 *vs*. control Caco-2 cells)

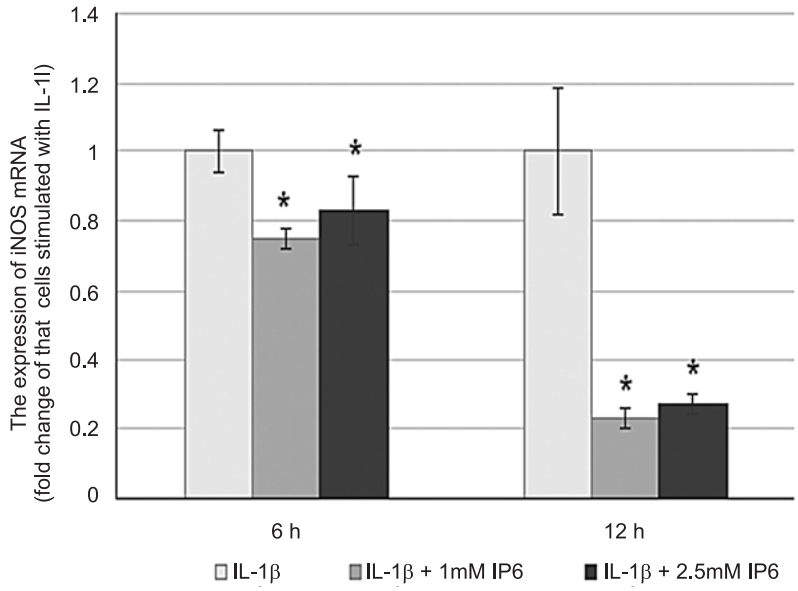

Figure 3. Changes in iNOS mRNA expression in Caco-2 cells treated with IL-1β and IP6 (1 and 2.5 mM) for 6 h and 12 h. The results are presented as the mean ± SD of three separate experiments; *p < 0.05 *versus* IL-1β-stimulated cells)

lating the production of proangiogenc factors (20-22). In colorectal cancer iNOS activity was correlated with increased VEGF expression and microvessel density (15). Furthermore, NO up-regulated MMP-2 and MMP-9 (matrix metalloproteinases), and down-regulated their inhibitors TIMP-2 and TIMP-3 (22). Thus, iNOS was suggested to play a crucial role in metastasis and cancer invasiveness, and metastatic tumors manifested its higher activity than non-metastatic ones (15).

Strategies aiming at inhibiting of iNOS gene expression and NO generation during chronic

inflammation may appear effective in decreasing the risk of cancer development in chronic inflammatory gastrointestinal diseases. Recently, many naturally ocurring phytochemicals have been shown to exert their anti-cancer properties through modulation of inflammatory responses (16, 20, 21). To date, various compounds from dietary sources including resveratrol (23-25), epigallocatechin-3-gallate (26), demethoxycurcumin (27) or proanthocyanidins from grape seeds (28) have been identified as inhibitors of iNOS. These plant derived products were observed to attenuate the synthesis of both mRNA and protein of iNOS and subsequent NO generation in different cell lines stimulated with

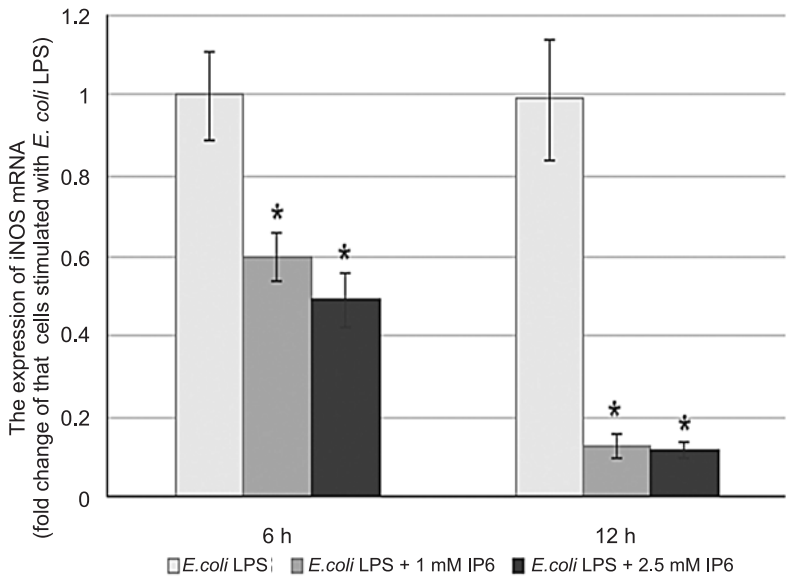

Figure 4. Changes in iNOS mRNA expression in Caco-2 cells treated with *E. coli* LPS and IP6 (1 and 2.5 mM) for 6 h and 12 h. The results are presented as the mean ± SD of three separate experiments; *p < 0.05 *versus* LPS-stimulated cells)

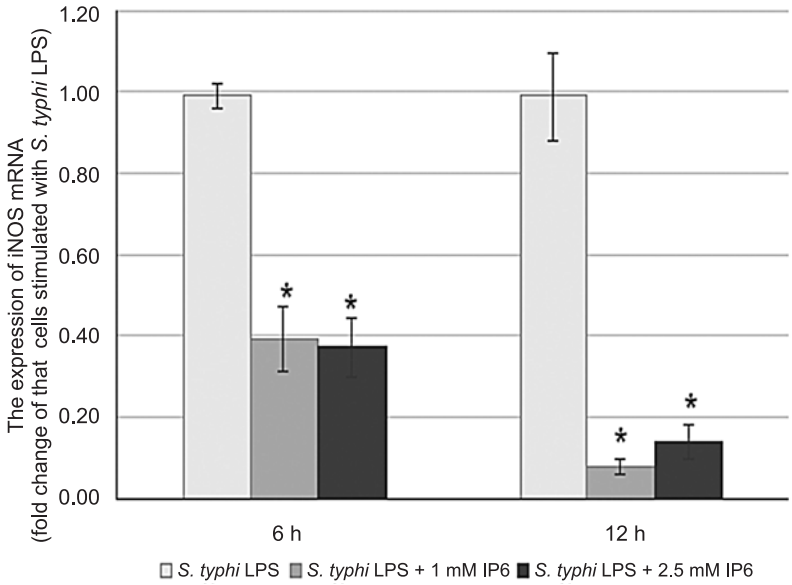

Figure 5. Changes in iNOS mRNA expression in Caco-2 cells treated with *S. typhi*. LPS and IP6 (1 and 2.5 mM) for 6 h and 12 h. The results are presented as the mean ± SD of three separate experiments; *p < 0.05 *versus* LPS-stimulated cells)

LPS and various cytokines (IL-1β, INF-γ). Inositol hexaphosphate, a bioactive constituent of high fiber diet (29) showed immunoregulatory and anti-inflammatory properties, which may contribute to its chemopreventive action (30-32). In the last years, direct and indirect effect of IP6 on the expression of nitric oxide synthases in *in vivo* models has been investigated (19, 33, 34). Liao et al. (19) showed that the activation of iNOS leads to prolonged production of NO in high, potentially cytotoxic concentrations. In tissues exposed to high concentrations of NO over long periods of time, such as in long-standing UC, genetic instability and accumulation of mutations could occur either through the direct action of NO or through the interaction of NO with superoxide to form highly reactive peroxynitrite. Nitrotyrosine, a biomarker of NO-mediated protein modification, is commonly used to detect NO-mediated cellular damage and its formation was reduced in mice treated with IP6. In terms of angiogenesis, the finding of Gu et al. (33) indicated that IP6 treatment of PC-3 tumor xenografts mice exerted potent antiangiogenic response by inhibiting the expression of both VEGF and eNOS, and subsequent NO-mediated signaling events. In turn, Raina et al. (34) evaluated a dose-dependent effect of IP6 TRAMP mice feeding on biomarkers associated with survival and angiogenesis in prostatic gland. The data indicated that IP6 significantly decreased iNOS expression in tumor tissues.

The aim of the present study was to evaluate the influence of IP6 on transcriptional activity of iNOS in Caco-2 cells in steady-state and inflammatory conditions. This is the first report on the potent inhibitory activity of IP6 on expression of this gene in an *in vitro* inflamed human intestinal cell model. In order to generate inflammatory microenvironment, Caco-2 cells were exposed to IL-1β and LPS derived from *Escherichia coli* and *Salmonella typhimurium*. The Caco-2 exhibited the basal expression of gene encoding inducible isoform of NOS and it has been enhanced with the exposure of cells to external stimuli. That effect was continued in a time-dependent manner. Our observation remains in agreement with the findings of Panaro et al. (23) and Somchit et al. (27), which have demonstrated the presence of iNOS mRNA in unstimulated Caco-2 cells and the increase of its level following IL-1β, TNF-α, INF-γ and LPS treatment. Jenkins et al. (35) showed that in human colon cancer DLD-1 cells contained iNOS mRNA regardless of whether or not the cells had been exposed to cytokines. In this case, it would appear that small amounts of RNA had been constitutively transcribed but that active enzyme was present only after its induction by LPS and/or cytokines. Our study indicated that IP6 inhibits constitutive and proinflammatory agents-induced transcriptional activity of iNOS gene in Caco-2 cells in time-dependent and dose-independent manner. Treatment of Caco-2 cells with IP6 alone resulted in significant inhibition of iNOS transcription. This effect was observed after long incubation with IP6 (12 h). Its inhibitory effect on iNOS transcription was more evident in cells stimulated with pro-inflammatory agents. Along with increasing duration of IP6 action on Caco-2 cells, the down-regulation of iNOS expression was stronger. It indicates that IP6 is able to counteract the response of intestinal cells to inflammation, probably by diminishing detrimental action of NO.

IP6 is suggested to exert anti-cancer influence on tumor cells *via* several mechanisms, including modulation of intracellular signaling cascades that involve phosphatidylinositol-3 kinase (PI3K), MAPK, PKC, AP-1, and NFκB (17, 18). IP6 was shown to inhibit NFκB activation in cervical cancer HeLa cells (36) and prostate cancer DU-145 cells (37). Also, our early studies demonstrated that IP6 modulated the expression of genes encoding the subunits of NFκB in the intestinal epithelial cells (31, 38). According to previously published data, IP6 was found to decrease the transcriptional activity of NF-κB, possibly resulting in decreased expression of its downstream targets, such as iNOS (33, 34).

The findings of this study suggest that one of the anti-cancer and anti-inflammatory abilities of IP6 can be realized by suppressing the expression of gene encoding inducible nitric oxide synthase isoform at the transcriptional level. Thus, iNOS could be another molecular target responsible for IP6 chemopreventive or chemoprotective action.

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