Inositol hexaphosphate (IP6) known as phytic acid is a naturally occurring polyphosphorylated carbohydrate with demonstrated antiproliferative and anticancer activity. It is abundant in the regular human diet (cereals and legumes) (1, 2). An anticancer function of IP6 has been demonstrated both in vivo and in vitro (3, 4). IP6 inhibited tumor growth and induced cell differentiation of several human cancer cell lines including colon (5, 6) mammary (7), prostate (8), laryngeal (9), hepatoma (10), pancreatic (11), and melanoma (12).

In the past years, much has been written regarding the mechanisms by which IP6 affected the growth of cancer cells. IP6 inhibited the growth of all most malignant cell lines, irrespective of whether they were epithelial or mesenchymal origin, however, cells from different origins have different sensitivities to IP6, suggesting that it affects different cell types through different mechanism of action. It has been shown that IP6 could regulate the cell cycle to block uncontrolled cell division and force malignant cells either to differentiate or go to apoptosis (5). It has also been shown that IP6 was able to modulate cellular response at the level of receptor binding. IP6, after sterically blocking the heparin-binding domain of basic fibroblast growth factor (bEGF), disrupted further receptor interactions (5, 13). In addition to blocking of phosphatidylinositil-3 kinase (PI3K) and activating protein-1 (AP-1) by IP6 (14), protein kinase C (PKC) (15) and mitogen-activated protein kinases (MAPK) (16) were involved in IP6-mediated anticancer activity. Moreover, it was recently shown that IP6 operated via a direct control of protein phosphorylation (17) and it could reduce carcinogenesis mediated by active oxygen species and cell injury via its antioxidative function (18). Additionally, it was reported that the ability of IP6 to arrest the cell cycle may be mediated by the transcriptional up-regulation of the p53-responsive p21WAF1 gene (19).

Although several mechanisms for IP6-mediated anticaner function have been proposed, very little supportive data exist to explain its effects on cytokine genes expression in cancer cells. The more so as IP6 present in the intestinal milieu may exert immunoregulatory effects on colonic epithelium under physiological conditions or during microbe-induced infection/inflammation (21). Additionally, it was reported that IP6 modulate cytokine production by human neutrophils (20) and colon cancer Caco-2 cells (21).

Pathological processes accompanying cancer colon diseases are associated with aberrant expression of many cytokines. Cancer cells release a mul-
titude of cytokines and growth factors that influence neighboring cells and help establish a favorable environment for tumor development. The cytokine profile in colon cancer indicates a strong unspecific inflammatory reaction in the tumor tissue represented by high level of TNF-α. TNF-α is a pleiotropic cytokine which is involved in numerous processes such as cell death and development, oncogenesis and immune, inflammatory, and stress response. It is intriguing that the cytokine possesses both growth stimulating properties and growth inhibitory properties (22). Most of the biological effects of TNF-α are mediated through the TNFR1 (p60) and TNFR2 (p80) receptors which are expressed on all somatic cells. TNFR1 has revealed the existence of so-called death domain, which is involved in signaling processes leading to programmed cell death. TNFR2 is responsible for activation of lymphocyte T and acute phase effect. Deregulation of the expression of genes coding for TNF-α and its receptors was observed in neoplastic diseases (23). The influence of phytic acid on TNF-α and its receptors genes’ expression in human colon cancer cells has not been a subject of investigation, so far.

Therefore in the present study, we investigated whether and how IP6 affects the transcriptional activation of TNF-α, TNFR1 and TNFR2 genes in Caco-2 cell lines by analyzing the amount of the corresponding mRNAs produced in the cells as a function of time of treatment and IP6 concentration.

**EXPERIMENTAL**

Caco-2 cell line was purchased from the German Collection of Microorganism and Cell Cultures (Braunschweig, Germany). Cells cultures were maintained at 37°C in 5% CO2 atmosphere in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES. They were treated with IP6 (Sigma) at the concentrations of 1, 2.5 and 5 mM for 1, 6, 12 and 24 h. As a control, Caco-2 cells were incubated under the same conditions but without stimulation with IP6. After the indicated time periods cells were collected, rinsed with phosphate-buffered saline and immediately used for total RNA extraction using TRIZol™ Reagent (Invitrogen). Quality and quantity of the isolated RNA was evaluated using agarose gel electrophoresis and optical density measurement at λ 260 nm, respectively. Transcriptional activity of the analyzed genes was assessed with the use of quantitative real-time PCR method (QRT-PCR) based on fluorescent TaqMan methodology. Commercially available gene specific fluorescent real-time PCR primers and probes (Applied Biosystems) were used. Reverse transcription and amplification reactions were performed on the ABI PRISM 7000 Detection System (Applied Biosystems). The conditions of one step RT-PCR reactions were as follows: 30 min at 50°C (RT), followed by 15 min at 95°C (DNA polymerase activation), and then 40 cycles of amplification for 15 s at 94°C and 1 min at 60°C. The threshold cycle (Ct) values were determined and normalized to the housekeeping gene β-actin, which was used as an endogenous control. Transcriptional activity of TNF-α and its receptors was expressed as a number of copies of their mRNAs per 1 µg of total RNA. All the results are presented as the means ± S.D. of two independent experiments, each performed in triplicate. Statistical analysis was performed with the use of STATISTICA ver. 6.0 software (StatSoft).

**RESULTS**

Experimental data showing the number of mRNA copies of TNF-α, TNFR1 and TNFR2 are demonstrated in Figures 1, 2 and 3, respectively. The analyzed genes were expressed in both non-stimulated cells as well as in cells stimulated with IP6 at different concentrations and for different time of stimulation. Control non-stimulated Caco-2 cells have shown the constant level of TNF-α mRNA (Figure 1) at all experimental conditions used, contrary to the mRNA level of TNFR1 and TNFR2 (Figures 2 and 3). An increase in both receptors mRNA level was seen with time increasing from 12 to 24 h in control cells. However, there was no statistical significance for those mRNAs with regard to time dependence and concentration fluctuation. Caco-2 cells shortly treated (6 h) with all of the used concentrations of IP6 have shown the level of expression of all analyzed genes to be very similar to that observed in control cells (Figures 1, 2 and 3).

After 12 h of incubation with IP6 cells responded with statistically significant decrease in TNF-α and TNFR2 expression for all experimental variation of IP6 concentration (Figures 1 and 3). Interestingly, an opposite effect was observed in cells treated for 12 h with IP6 at 2.5 and 5 mM, in this set of samples the increased level of TNFRI mRNA was detected but only for 5 mM IP6 this increase was statistically significant (p = 0.0147) when compared to the control (Figure 2). It was also found, that at 24 h the highest concentration of IP6 (5 mM) evoked marked stimulation of TNF-α and
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Figure 1. Bifactorial variation analysis of the number of TNF-α mRNA copies calculated per 1 µg of total RNA isolated from control (K) and treated with 1, 2.5 and 5 mM IP6 for 1, 6, 12 and 24 h Caco-2 cells.

Figure 2. Bifactorial variation analysis of the number of TNFRI mRNA copies calculated per 1 µg of total RNA isolated from control (K) and treated with 1, 2.5 and 5 mM IP6 for 1, 6, 12 and 24 h Caco-2 cells.

Figure 3. Bifactorial variation analysis of the number of TNFRII mRNA copies calculated per 1 µg of total RNA isolated from control (K) and treated with 1, 2.5 and 5 mM IP6 for 1, 6, 12 and 24 h Caco-2 cells.
TNFRII genes transcription, as reflected by statistically significant difference in mRNAs level over that in control cells (p = 0.0054 and p = 0.0006, respectively). On the contrary, the level of TNFRI gene expression decreased to the control amount under influence of 5 mM IP6 at 24 h (Figure 2).

DISCUSSION AND CONCLUSIONS

The obtained results let us conclude that phytic acid has an influence on the level of TNF-α gene expression, as well as on the level of expression of genes coding for two TNF-α receptors. Lower concentrations of IP6 (1 and 2.5 mM) and short incubation time (up to 6 h) did not elicit strong impact on the transcription of all analyzed genes, while the highest concentration of IP6 (5 mM) used in the study up-regulated the transcription of TNF-α and TNFRII at 24 h incubation. The increase in transcription of TNFRI at 24 h was followed by its down-regulation with prolongation of cells exposure to IP6 up to 24 h. Statistically significant increase in TNFRI mRNA level for cell samples stimulated with 5 mM IP6 for 12 h might be discussed as the reflection of apoptotic effect of IP6, especially that TNFRI is well known programmed cell death inducer (5). On the other hand, relatively lower level of TNF-α and its second receptor (TNFRII) mRNAs in cells treated with IP6 for 12 h might be discussed as the reflection of apoptotic effect of IP6.

According to Weglarz et al. (21) IP6 had a suppressive effect on IL-8 secretion by LPS stimulated colonocytes and this might be one of theoretical explanations of anti-inflammatory activity of IP6 in the intestine. Cheng et al. (24) additionally proved, that IP6 decreased IFN-γ and IL-10 secretion, however, they used different in vitro model, that is peripheral blood mononuclear cells, to study the effect of IP6 on cytokine production. Also interesting is the described by Sveinbjörnsson group (25) relation between TNF-α, TNFRI, IFN-γ and apoptosis of mouse colon cancer cell line C-26. The above-mentioned research group showed that cytotoxic effect of TNF-α on C-26 cells depended on the activation of TNFRI. Moreover, C-26 cells apoptosis was induced by TNF-α only in the presence of IFN-γ. To sum up, IP6 in Caco-2 cells, through regulation of cytokine production, such as TNF-α, IL-8 or IFN-γ, may modulate not only processes directly involved in the immune response (e.g. inflammatory process), but it also may play a role in cell death activation or cell survival. A role of IP6 in apoptosis control is also described by the findings that inositol hexaphosphate kinase-2 is a physiological mediator of cell death from the one hand, but from the other one, IP6 was shown to protect rat mesencephalic cells N-27 from iron-induced apoptosis (5).

To find clear explanation of IP6 action on Caco-2 colon cancer cells in terms of cytokine production regulation, further studies have to be scheduled. Future studies should basically be directed to analyze the changes in gene expression not only at the transcription level, but also at the protein synthesis and protein–protein interaction level.

REFERENCES