Colorectal cancer is the second most common cause of cancer incidence and death in both men and women in Europe (1). Epidemiological data indicate that the life-style and dietary habits have been identified as major risk factors for colon cancer growth and progression. Several naturally occurring dietary phytochemicals have revealed a chemopreventive and therapeutic effect against cancer (2, 3).

Phytic acid, a hexaphosphorylated inositol (IP6) is a major fiber-associated component of wheat bran and legumes. A number of recent studies revealed strong anti-cancer activity of IP6, and hence, the unknown molecular mechanisms of its action are still a subject of investigation. Dietary phytic acid can be internalized by the cells, included into cellular inositol phosphate pool, dephosphorylated to IP1-5 which further can act as secondary messengers (4, 5). Therefore, IP6 is hypothesized to target cancer through multiple pathways, i.e., modulation of cell signal transduction, inhibition of cell proliferation, and cell cycle progression, activation of apoptosis, induction of cell differentiation and anti-angiogenic potential (6-10).

Nuclear factor κB (NF-κB), a member of transcriptional factor family, plays an important role in regulating the expression of genes involved in a number of cellular processes (11-13). Constitutively activated NF-κB is associated with many aspects of tumor development, including promoting cancer cell proliferation, preventing apoptosis and increasing metastatic potential (14, 15). The mammalian NF-κB family consists of five proteins: c-Rel, p50 (NF-κB1), p65 (RelA), p52 (NF-κB2), and RelB, which have Rel homology domain (RHD) at N-terminal sequence. All members of this family create a various homo- and heterodimers which activate NF-κB-dependent genes. In the cytoplasm of unstimulated cells the predominant dimmer of NF-κB is an inactive p50/p65 heterodimer complexed with the inhibitor IκBα (16, 17). External and internal stimulations via proinflamatory cytokines, growth factors, oxidative stress, ionizing radiation and chemotherapeutic agents can activate the NF-κB.

EVALUATION OF THE EXPRESSION OF TRANSCRIPTIONAL FACTOR NF-κB INDUCED BY PHYTIC ACID IN COLON CANCER CELLS

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Abstract: Over the last years phytic acid, a hexaphosphorylated inositol (IP6) has attracted particular attention due to its anti-cancer activity, however, the molecular mechanisms of its action have not been elucidated, as yet. The aim of this study was to evaluate the influence of phytic acid on the expression of genes encoding p65 and p50 subunits of NF-κB and of its inhibitor IκBα in human colorectal cancer cell line Caco-2. A kinetic study of p65 and p50 subunits and IκBα mRNAs expression was performed on Caco-2 cells after treatment with 1, 2.5 and 5 mM IP6 for 1, 6, 12 and 24 h. Quantification of the genes expression was carried out using real time QRT-PCR technique. Treatment of cells with 5 mM IP6 resulted in a strong increase in IκBα expression at 6 h, 12 h and 24 h. The level of p65 transcript after 1 h was lower in the cells exposed to 1, 2.5, and 5 mM IP6 than in the control cells. The increase in transcriptional activity of p65 gene in response to 5 mM IP6 after 6 h and 12 h was observed. Cells treated for 24 h with 2.5 mM and 5 mM IP6 showed a significant decrease in expression of p65 gene. There were no quantitative changes in the p50 gene expression in the cells treated with IP6 compared to the control cells. High positive correlation between the expression of IκBα and p65 was detected. The results of this study suggest that IP6 primarily influences p65 and IκBα genes expression in colon cancer cells. Changes in transcriptional activities of IκBα and p65 depend on IP6 concentration and time of its action.

Keywords: phytic acid, NF-κB, colon cancer, real-time QRT-PCR, mRNA quantification

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pathway (18). The IκBα kinase complex (IKK) catalyzes the phosphorylation of IκB, which stimulates its ubiquitination and subsequent degradation. The released NF-κB then translocates to nucleus, binds to specific DNA sequences and activates transcription of target genes (16).

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**EXPERIMENTAL**

**Cell culture**

The study was performed on an *in vitro* model using the Caco-2 human colon adenocarcinoma cell line (DSMZ, Braunschweig, Germany). The cells were cultured in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (GibcoBRL), 100 µg/mL penicillin (Sigma Aldrich), 100 µg/mL streptomycin (Sigma Aldrich) and 10 mM HEPES (GibcoBRL). They were grown at 37°C as monolayers in a humidified atmosphere containing 5% CO2. Cells were treated with 1, 2.5 and 5 mM IP6 (pH 7.4) (Sigma Aldrich) for 1, 6, 12 and 24 h. As a control, Caco-2 cells were incubated under the same conditions without stimulation with IP6.

**RNA extraction**

Total RNA was extracted from control and IP6 treated cells with the use of TRIZOL® reagent (Invitrogen) according to the producer’s protocol. The RNA extracts were qualitatively checked by electrophoresis in 1.0% agarose gel stained with ethidium bromide. RNA concentration was determined spectrophotometrically using a GeneQuant Pro (Amersham Biosciences) spectrophotometer.

**Real-time QRT-PCR assay**

Transcriptional activity of p50, p65 and IκBα genes was evaluated on the basis of copy number of mRNA related to 1 mg of total RNA by the use of real time QRT-PCR technique with a SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen). The analysis was carried out using an Opticon™ DNA Engine Sequence Detector (MJ Research, USA). Oligonucleotide primers specific for p50, p65, IκBα mRNAs were chosen based on the literature (19). QRT-PCR assay was performed in triplicate for each sample. 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 45s, for amplification. After completion of the cycle process, the samples were subjected to temperature ramp from 60°C to 95°C at the rate of 0.2°C/s with continuous fluorescence monitoring for melting curve analysis. β-Actin was used as an internal control in each single QRT-PCR for all samples. 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). Finally, specificity of RT-PCR reaction was confirmed by determining the characteristic temperature of melting for each amplimer, i.e., p50 – 78°C; p65 – 85°C; IκBα – 87°C. RT-PCR products were separated on 6% polyacrylamide gel (PAA) and visualized with silver staining.

**Statistical analysis**

Statistical analysis was performed with the use of Statistica PL 6.0 software. All the results were expressed as the means ± S.D. The one-way ANOVA and 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. Correlations were evaluated using the Spearman’s rank correlation test. Significance level was assumed for p < 0.05.

**RESULTS**

In the present study, mRNAs of p50 and p65 subunits of NFκB and that of its inhibitor IκBα in colon cancer cells treated with phytic acid using real-time QRT-PCR assay were determined.

Specificity of RT-PCR for the target genes was experimentally confirmed by PAA electrophoresis and the amplimers melting temperatures. For each RT-PCR product, a single peak was obtained by melting curve analysis at the expected temperatures, i.e., p50 – 77.6 ± 0.27°C ; p65 – 84.8 ± 0.29°C ; IκBα – 86.3 ± 0.28°C (Fig. 1A). Gel electrophoresis revealed the presence of single products of the predicted size (p50 – 262 bp; p65 – 223 bp; IκBα – 218 bp) (Fig. 1B).

The expression of p50, p65 and IκBα mRNAs was found both in the control cells and in all the cells treated with different doses of IP6 for 1, 6, 12 and 24 h. There were no statistically significant changes in the p50 gene expression in the cells treated with IP6 compared to the control cells (p > 0.05; ANOVA) (Fig. 2A). The level of p65 transcript after 1 h was significantly lower in the cells exposed to 1 mM (1.0 × 10² ± 0.11 mRNA p65 copies per 1 µg RNA), 2.5 mM (1.1 × 10² ± 0.09) and 5 mM IP6 (1.0 × 10² ± 0.26) than in the control cells (1.6 × 10² ±
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There was no statistically significant difference between p65 mRNA quantities in the cells exposed to increasing concentrations of IP6 for 1 h. At 6 h, in all cell cultures incubated with IP6 the increase of p65 mRNA expression was observed. However, the transcriptional activity of p65 gene in response to 1 mM (1.7 × 10⁵ ± 0.06) as well as to 5 mM IP6 (1.8 × 10⁵ ± 0.05) was markedly higher than in control cells (1.4 × 10⁵ ± 0.04) (p < 0.05) and cells incubated with 2.5 mM IP6 (1.5 × 10⁵ ± 0.13) p65 mRNA copies per 1 mg RNA) exhibited its higher expression, nevertheless with no significant difference. After 12 h of incubation with the highest concentration of IP6 the level of p65 mRNA (2.1 × 10⁵ ± 0.26) exceeded that of control cells (1.6 × 10⁵ ± 0.21) (p = 0.048). The level of p65 gene expression in Caco-2 cells exposed to 1 mM (1.5 × 10⁵ ± 0.10) and 2.5 mM IP6 (1.4 × 10⁵ ± 0.03) was insignificantly lower than in untreated cells (p > 0.05). Cells treated for 24 h with 1 mM (1.8 × 10⁵ ± 0.17) (p > 0.05), 2.5 mM (1.3 × 10⁵ ± 0.26) (p = 0.0026) and 5 mM IP6 (1.5 × 10⁵ ± 0.13) (p = 0.0112) showed a decrease in p65 gene expression in comparison to control cells (2.3 × 10⁵ ± 0.22) (Fig. 2B).

Gene encoding IκBα inhibitor was found to be expressed at the similar level (p = 0.7353; ANOVA) in both non-stimulated as well as in cells stimulated with IP6 at different concentrations for 1 h. After 6 h, transcriptional activity of IκBα tended to rise with increasing IP6 doses. Comparative analysis of IκBα mRNA copies per 1 mg of total RNA revealed

Figure 1A. Melting curve of amplification products of p50, p65 and IκBα.
Figure 1B. Detection of RT-PCR products by 6% PAA electrophoresis; lanes 1, 2 – IκBα (218 bp); lanes 3, 4 – p65 (223 bp); lanes 5, 6 – p50 (262 bp); lane 7: size marker pBR 322/HaeIII.
higher, although not statistically significant quantities in response to 1 mM IP6 \((8.2 \times 10^4 \pm 1.36)\) as compared to untreated cells \((6.1 \times 10^4 \pm 0.59)\) \((p > 0.05)\). The markedly higher level of I\(\kappa\)B\(\alpha\) transcription was induced by 2.5 mM \((9.2 \times 10^4 \pm 1.27)\) as well as by 5 mM IP6 \((1.2 \times 10^5 \pm 0.13)\) \((p = 0.0009)\) than in control cells. At 12 h incubation, the I\(\kappa\)B\(\alpha\) inhibitor did not show any significant changes in transcription in response to 1 mM \((5.8 \times 10^4 \pm 0.13)\) and 2.5 mM IP6 \((7.5 \times 10^4 \pm 0.61)\) in relation to the control cells \((6.5 \times 10^4 \pm 0.52)\) \((p > 0.05)\). A similar trend was also observed after 24 h, as indicated by mRNA copy numbers: control: \(9.3 \times 10^4 \pm 0.91\); 1 mM IP6: \(9.1 \times 10^4 \pm 0.93\); 2.5 mM IP6: \(7.4 \times 10^4 \pm 0.42\) \((p > 0.05)\). Treatment of cells with 5 mM IP6 resulted in a strong increase in I\(\kappa\)B\(\alpha\) expression at 12 h \((1.4 \times 10^5 \pm 0.16; p = 0.0002)\) and 24 h \((1.3 \times 10^5 \pm 0.08; p = 0.0011)\) (Fig. 2C).

Furthermore, high positive correlation between the expression of I\(\kappa\)B\(\alpha\) and p65 genes \((R = 0.72, p = 0.000; \text{Spearman’s rank correlation test})\) was observed, whereas no correlations between I\(\kappa\)B\(\alpha\) and p50 \((R = 0.37, p = 0.1465)\) and between p50 and p65 transcript levels \((R = 0.39, p = 0.1220)\), could be demonstrated.

**DISCUSSION and CONCLUSION**

The nuclear factor \(\kappa\)B plays an essential role in regulating a wide variety of gene expression, including those involved in cell cycle regulation, proliferation, differentiation, apoptosis and other processes. Disturbances in NF-\(\kappa\)B signaling pathway are known to contribute to pathogenesis of various diseases, like a cancer. Several studies in recent years have shown inhibitory effect of dietary supplements on various signaling cascades including NF-\(\kappa\)B \((2, 20)\).

In the present study we evaluated the influence of phytic acid on transcriptional activity of p50, p65 and I\(\kappa\)B\(\alpha\) genes in human colon cancer cells. In many types of human tumors, e.g., lymphoma \((21)\), gastric carcinoma \((22)\), pancreatic adenocarcinoma \((23)\), NF-\(\kappa\)B is characterized by its constitutive activation. In colon cancer tissue \((24)\) and in cell lines (HT-29, SW48, RKO) \((14, 25, 26)\) the transcriptional factor continual expression have been shown too. We have detected mRNAs of the studies genes in control cells cultured for 1, 6, 12 and 24 h, and, NF-\(\kappa\)B expression was also found out in cells treated with IP6 at all tested conditions. Yu et al. \((24)\) showed that Rel A subunit of NF-\(\kappa\)B was activated in human colorectal tumor tissue, but not in normal tissue. Therefore, it was of our interest to analyze the effect of IP6 on sustained NF-\(\kappa\)B expression in the human Caco-2 cell line.

Heterodimer p65/p50 is the most common NF-\(\kappa\)B complex in cells. The Rel family (p65) promotes transcription of NF-\(\kappa\)B-dependent genes, because it contains a transactivation domain (TAD). P50-homodimer lacks TAD domain, and hence, has no intrinsic ability to induce genes transactivation. However, its binding to \(\kappa\)B sites in the promoter regions of genes leads to repression of their expression \((16)\). Biological role of NF-\(\kappa\)B is determined by Rel family proteins interaction with its inhibitors, therefore NF-\(\kappa\)B expression has commonly been
evaluated in the context of both its and IκBα transcriptional activities.

The findings of the present study revealed that IP6 at all used concentrations did not cause any alterations in p50 gene expression in Caco-2 cells. However, time dependent changes in transcriptional activities of IκBα and p65 could be detected in both control cells and in cells incubated with IP6. Treatment with 5 mM IP6 of cells for 1 h resulted in a decrease in the level of p65 mRNA, whereas IκBα transcripts remained at the same level. In response to IP6 stimulation for 6 h an increase in transcriptional activity of p65 and IκBα was observed with the IκBα mRNA level increasing proportionally with IP6 increasing concentration. A long term incubation (24 h) with IP6 evoked down-regulation of p65 gene transcription in Caco-2 cells and a strong activation of IκBα gene by 5 mM IP6.

In similar studies reported by Agrawal et al. (27) IP6-treated prostate cancer cells DU 145 showed a decrease in nuclear levels of p65 and p50 proteins. Inhibition of NF-κB activation was observed in cells treated with 1 mM IP6 for 12 h, and a decrease in its expression was more pronounced after 24 h incubation. The lowest activity of nuclear factor κB was detected in cells exposed to 2 mM IP6 for 24 h. Furthermore, it has been shown that IP6 evoked a significant increase in total IκBα protein level.

The kinetics of p65 gene expression may be determined via negative-feedback regulation of signaling pathway since the transcription factor controls expression genes that encode negative regulators of NF-κB (17). IκB proteins are known as the regulators of NF-κB activity. The results of the present study proved that increased transcriptional activity of the p65 gene was associated with increased transcriptional activity of IκBα gene, what may suggest co-expression of these genes. Mori et al. (21) detected higher expression of IκBα mRNA with no concomitant increase in IκBα protein level in adult T-cell leukemia cells. According to those authors, the increased turnover of the IκBα protein was associated with the activation of NF-κB. They suggested that the degradation of IκBα might play a role in the constitutive activation of NF-κB (21). The studies by Ferry et al. (28) indicated that IP6 could inhibit the Akt/NF-κB cell survival pathway. Our previous study revealed significant inhibition of Caco-2 cells growth by 5 mM IP6 (29), hence, based on the present results it can be suggested that the ability of 5 mM IP6 to inhibit colon cancer cells proliferation may be mediated through the stimulation of IκBα expression at the mRNA level.

In conclusion, phytic acid appears to alter p65 and IκBα genes expression in colon cancer cells which can explain its biological relevance in causing inhibition of cancer cell proliferation.

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