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Quantitative analysis of the level of *p53* and *p21*^{WAF1} mRNA in human colon cancer HT-29 cells treated with inositol hexaphosphate

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The aim of this study was to analyze the molecular mechanism of inositol hexaphosphate ($InsP_6$) action through which it may inhibit proliferation of colon cancer cells and cell cycle progression. A kinetic study of *p53* and *p21*^{WAF1} mRNA increase was performed on human colon cancer HT-29 cells after treatment with 1, 5 and 10 mM $InsP_6$ for 6, 12, 24 and 48 h. Real-time-QPCR based on TaqMan methodology was applied to analyze quantitatively the transcript levels of these genes. The transcription of β-actin and GAPDH genes was assessed in parallel to select the control gene with least variability. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene transcription. $InsP_6$ stimulated *p53* and *p21*^{WAF1} expression at the mRNA level, with the highest increase in *p21*^{WAF1} mRNA occurring at 24 h, i.e., following the highest increase in *p53* mRNA observed at 12 h. Based on these studies it may be concluded that the ability of $InsP_6$ to arrest the cell cycle may be mediated by the transcriptional up-regulation of the p53-responsive *p21*^{WAF1} gene.

INTRODUCTION

Inositol hexaphosphate ($InsP_6$), also known as phytic acid, is a natural dietary component, especially abundant in cereals, legumes, oil seeds and wheat bran, as a major fiber-associated component (Graf & Eaton, 1985; Shamsuddin *et al.*, 1997; Jenab & Thompson, 2000). Several physiological activities have been recognized for $InsP_6$ (Regunathan *et al.*, 1992; Vucenik *et al.*, 1999; Grases & Costa-Bauza, 1999), however, over the last years it has attracted particular attention due to its anti-neoplastic potential. *In vitro* and *in vivo* studies have demonstrated both chemopreventive and anti-carcinogenic effects of $InsP_6$ against colon (Pretlow *et al.*, 1992; Sakamoto *et al.*, 1993; Shamsuddin *et al.*, 1997), skin (Ishikawa *et al.*, 1999), mammary (Shamsuddin & Vucenik, 1999), prostate (Shamsuddin & Yang, 1995), liver (Vucenik *et al.*, 1998) and lung (Wattenberg, 1999)

tumorigenesis. Studies on human and rodent cancer cell lines showed that $InsP_6$ reduces cellular proliferation rate and DNA synthesis with the enhancement of differentiation of malignant cells to a more mature phenotype, sometimes resulting in reversion to normal (Yang & Shamsuddin, 1995; Shamsuddin *et al.*, 1996). $InsP_6$ has been found to cause G_1 cell cycle arrest in mammary cancer cell lines MCF-7 and MDA-MB 231, and in HT-29, a human colon cancer cell line (El-Sherbiny *et al.*, 2001).

The basic mechanism of the anti-carcinogenic effect of $InsP_6$ is still under investigation. It has been shown to inhibit cell transformation by targeting phosphatidylinositol-3-kinase (PI3-K) in JB6 mouse cells (Huang *et al.*, 1997) and to block transforming growth factor α-induced binding of activated ErbB1 to AP2 in human prostate cancer DU145 cells (Zi *et al.*, 2000). It has also been shown to activate apoptotic machinery and inhibit AKT/NFκB-mediated survival

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in HeLa cells (Ferry *et al.*, 2002). Treatment of MCF-7 human breast cancer cells with $InsP_6$ caused an increase in the expression of anti-proliferative PKCδ (Vucenik *et al.*, 2005). Singh *et al.* (2003) reasoned that cyclin-dependent kinase inhibitors (CDKIs) and pRb-related proteins could be tumor suppressor targets for $InsP_6$ in modulating cell cycle progression in advanced prostate cancer. CDKI $p21^{WAF1}$ is an important effector that acts by inhibiting CDK activity in p53-mediated cell cycle arrest in response to various agents (Dulic *et al.*, 1994). Increased expression of $p21^{WAF1}$ may play a crucial role in the G_1/S phase arrest induced in transformed cells and may prevent the progression of neoplasia (Kim *et al.*, 2001).

In the present study, we investigated whether and how $InsP_6$ affects the transcriptional activation of *p53* and $p21^{WAF1}$ genes in human colon cancer cells, by analyzing the amount of the corresponding mRNAs produced in the cells as a function of time of treatment and $InsP_6$ concentration. A real-time reverse transcriptase-polymerase chain reaction (real-time-PCR) assay based on TaqMan methodology was applied for detection and quantification of *p53* and $p21^{WAF1}$ mRNAs.

MATERIALS AND METHODS

Cell culture. The HT-29 human colon carcinoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. They were grown at 37°C as monolayers in a humidified atmosphere containing 5% CO_2. Cells (1×10^6) were seeded in 21.5 cm^2 culture flasks and after 3 days of growth they were treated with 1, 5 or 10 mM $InsP_6$ (pH 7.4) dissolved in distilled water, for 6, 12, 24 and 48 h. As a control, HT-29 cells were incubated under the same conditions but without stimulation with $InsP_6$. After incubation for the indicated time, the monolayers were rinsed with cold phosphate-buffered saline and lysed for 5 min with the use of TRIzol reagent (Life Technologies) and the lysates were then subjected to the extraction of RNA.

RNA isolation and real-time-QPCR assay. Total cellular RNA was extracted from the cell lysates according to the manufacturer's protocol (Life Technologies). The *p53* and $p21^{WAF1}$ genes expression was detected by real-time-QPCR assay. Quantification of *p53* and *p21* mRNA was achieved by means of the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). RT-PCR was based upon the TaqMan fluorogenic detection system (TaqMan®, PE Applied Biosystems), using a fluorogenic oligonucleotide probe designed to hybridize to the specific target sequence. The TaqMan

probes were labeled at the 5′ end with the fluorescent reporter dye FAM (6-carboxyfluorescein) (R) and at the 3′ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine) (Q). The sequences for gene-specific forward and reverse primers and the probes were designed using Primer Express 1.0 software (PE Applied Biosystems). The following sequences of primers and probe were used for RT-PCR of *p53* mRNA: 5′ TAACAGTTCCTGCATGGGCG-GC 3′ (forward); 5′ AGGACAGGCACAAACACG-CACC 3′ (reverse); 5′ CGGAGGCCCATCCTCAC-CATCATCA 3′ (probe) (GeneBank, Accession No. NM_000546). The following primers and probe were used for RT-PCR of $p21^{WAF1}$ mRNA: 5′ CACTC-CAAACGCCGGCTGATCTTC 3′ (forward); 5′ TG-TAGAGCGGGCCTTTGAGGCCCTC 3′ (reverse); 5′ CCAAGAGGAAGCCCTAATCCGCCCACAGGA 3′ (probe) (GeneBank, Accession No. NM_078467). In one-step RT-PCR, reverse transcriptase (MultiScribe, PE Applied Biosystems) and AmpliTaq Gold DNA polymerase (PE Applied Biosystems) were used. RT-PCRs were performed in a MicroAmp optical 96-well plate. Ten microliters of reaction mixture were used, containing 100 ng of total RNA, 1x TaqMan buffer, 3.5 mM $MgCl_2$, 200 μM each of dATP, dGTP and dCTP, 600 μM dUTP, 0.2 μM forward and reverse primers, 0.1 μM TaqMan probe, 0.5 U of MultiScribe and 0.5 U of AmpliTaq Gold. The conditions of one-step RT-PCR were as follows: 30 min at 60°C, 10 min at 95°C, and then 45 cycles of amplification for 15 s at 95°C and 1 min at 55°C followed by terminal elongation for 10 min at 72°C. Triplicate RT-PCR reactions were prepared for each sample. The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (C_t), was determined for each sample, and the average C_t of triplicate samples was calculated. For characterization of the generated amplicons and to control for contamination by unspecific byproducts, a melting curve analysis was applied between 60–95°C at 1°C intervals with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) using the fluorescent dye SYBR Green. In addition, the PCR products' size was verified by electrophoresis on 6% polyacrylamide gel with silver staining and comparing their mobilities with those of molecular mass marker pBR322/HaeIII (MBI Fermentas) based on the program GelScan version 1.45.

Endogeneous controls for target gene expression evaluation. The most commonly used housekeeping genes, β-actin and GAPDH (Bas *et al.*, 2004), were used to select a suitable endogenous control that gives the most reproducible results under our experimental conditions. The transcription of both genes in HT-29 cells incubated with $InsP_6$ at the concentrations of 1, 5 and 10 mM for 6, 12, 24 and 48 h, and in cells incubated without $InsP_6$ was studied

by RT-PCR. The PCR primers and the probes for β-actin and GAPDH were purchased from PE Applied Biosystems. The sequences of the two pairs of primers were as follows: 5′ AGCATCTAACCCGTGTCACACCCACT 3′ (forward), 5′ GGAACCGTTACCGGCAAGGCGAC 3′ (reverse) for β-actin, and 5′ GAAGGTGAAGGTCGGAGTC 3′ (forward), 5′ GAAGATGGTGATGGGATTTC 3′ (reverse) for GAPDH. The following probes were used: 5′ TGCGTCCTACCGTACCCCCTCCCGTA 3′ (for β-actin) and 5′ CCGACTCTTGCCCTTCGAAC 3′ (for GAPDH). The probes were labeled with the 5′ FAM reporter dye and the 3′ TAMRA quencher dye. The control genes were amplified in triplicate under conditions applied for the amplification of the target genes.

Quantification of expression of housekeeping genes. To quantify the results obtained by RT-PCR for β-actin and GADPH, the standard curve method was used. A commercially available standard of β-actin (TaqMan DNA Template Reagent Kit P/N 401970, PE Applied Biosystems) was amplified at five different DNA template concentrations: 0.6, 1.2, 3.0, 6.0, 12.0 ng/μl. Values of copy numbers for the standards were calculated based on the relationship that one 1 ng of DNA is equal to 333 genome equivalents (TaqMan PCR Reagent Kit Protocol P/N 402823). Amplification plots for each dilution of control template were used to determine the C_t value. A standard curve was generated by plotting the C_t values against the log of known input DNA copy numbers.

Quantification of expression of target genes. To determine the quantity of the target gene-specific transcripts present in treated cells relative to untreated ones, their respective C_t values were first normalized by subtracting the C_t value obtained from the β-actin control ($\Delta C_t = C_{t,}$ target $- C_{t,}$ control). The concentration of gene-specific mRNA in treated cells relative to untreated cells was calculated by subtracting the normalized C_t values obtained for untreated cells from those obtained from treated samples ($\Delta\Delta C_t = \Delta C_{t,}$ treated $- \Delta C_{t,}$ untreated) and the relative concentration was determined ($2^{-\Delta\Delta Ct}$).

The experiments where molecular methods were applied were carried out at the Department of Molecular Biology and Medical Genetics of the Medical University of Silesia.

Statistical analysis. In the analysis of control gene expression, the Kruskal-Wallis statistics was used to find the significance of differences between the groups within individual incubation times. The significance of differences between mean values of non-parametric data allowing one to find kinetic changes in transcription of control genes was analyzed by U Mann-Whitney test. Statistical significance of the target gene transcription changes was performed with the use of Tukey test. All the results

are expressed as means ±S.D. representing three independent experiments, each performed in triplicates. A P value of < 0.05 was considered statistically significant. Statistical analysis was performed with the use of the computer program Statistica PL V 6.0.

RESULTS

Confirmation of primer specificity

Real-time PCR for the control and target genes were performed in parallel for each experimental sample. Figure 1 shows real-time-QPCR amplification plots of β-actin, GAPDH, *p53* and *p21^{WAF1}* gene transcripts in InsP$_6$-untreated (control) cells. The specificity of real-time-QPCR amplification for the two target genes was confirmed by melting curve analysis and resulted in single product specific melting temperatures, as follows: *p53* — 81.3°C and *p21^{WAF1}* — 82.6 °C (Fig. 2). Gel electrophoresis revealed the presence of single products with the desired lengths (β-actin — 293 bp; *p53* — 121 bp; *p21^{WAF1}* — 101 bp) (Fig. 3). No primer-dimers or unspecific byproducts were generated during the 45 real-time PCR amplification cycles applied.

Validation of internal control genes

The mRNA starting copy numbers of both reference genes obtained from a standard curve based on C_t values and related to 1 μg of total RNA in HT-29 cells incubated with 1 mM, 5 mM and 10 mM InsP$_6$ for 6, 12, 24 and 48 h and in control cells incubated for the same time points are presented in Fig. 4. Statistical analysis of the experimental data
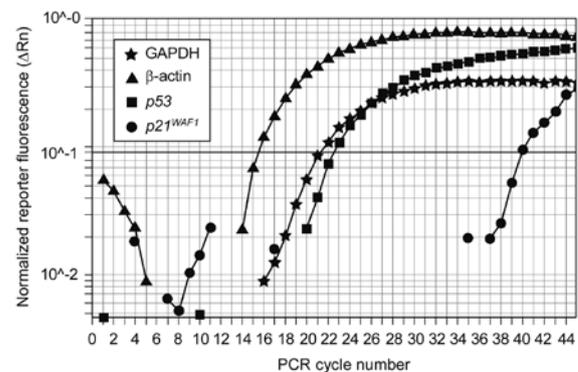

Figure 1. Amplification plots for GAPDH, β-actin, *p53* and *p21^{WAF1}* of control HT-29 cells by real-time PCR based on TaqMan fluorogenic system.
ΔRn represents fluorescence signal of the reporter dye divided by the fluorescence of the quencher dye minus baseline fluorescence. Amplification curves were used to determine the C_t values.
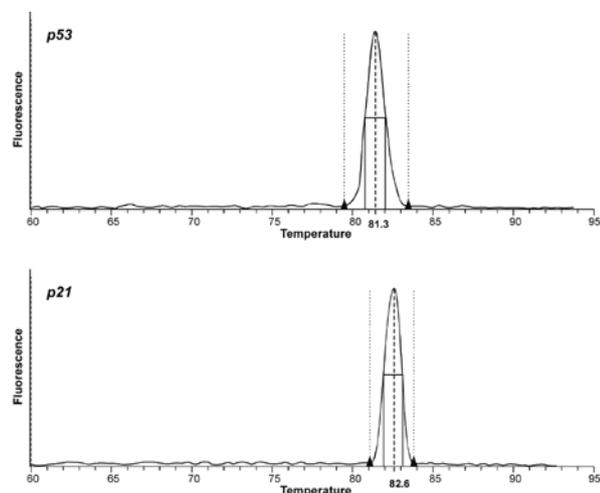
Figure 2. First derivatives of melting curves of amplification products of *p53* and *p21^WAF1*.

with the use of Kruskal-Wallis test revealed that in cells treated with different $InsP_6$ concentrations at each of three time points (6, 12 and 24 h), β-actin copy numbers were more constant ($P > 0.05$) compared to those of GAPDH. Only at the 48 h incubation were significant fluctuations in β-actin mRNA levels monitored in cells in the presence of 1, 5 and 10 mM $InsP_6$ ($P < 0.05$). β-Actin was more variable than GAPDH in its basal expression as a function of time, as confirmed by statistically significant differences ($P < 0.000032$). In contrast, GAPDH expression showed statistically significant differences ($P < 0.05$) when considered both at each time point following treatment with increasing $InsP_6$ concentrations and as a function of incubation period (Fig. 4).

Although the housekeeping genes should not be influenced by the experimental treatment, according to the literature data all housekeeping genes tested so far are either more or less regulated (Blanquicett *et al.*, 2002), and their transcription in a living
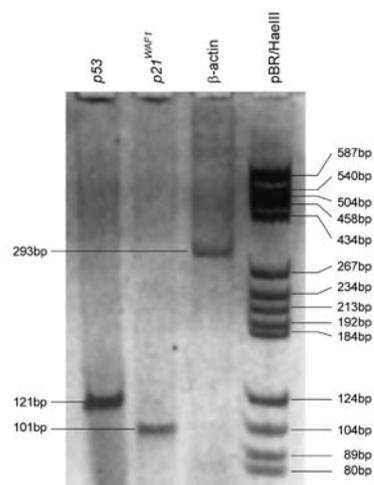

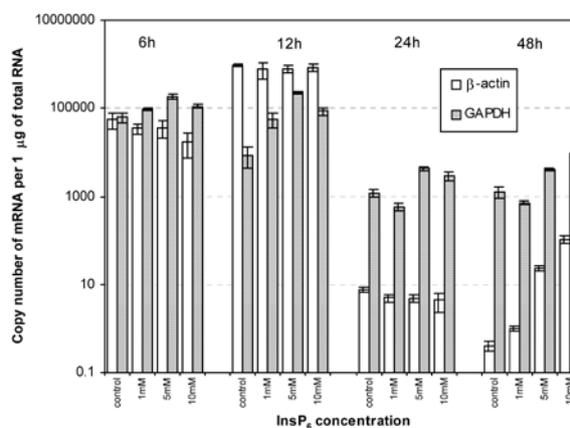**Figure 3. Detection of RT-PCR products by electrophoresis in 6% polyacrylamide gel.**

Figure 4. Absolute copy numbers of β-actin and GAPDH mRNAs in HT-29 control cells and in cells incubated with 1, 5 and 10 mM $InsP_6$ for 6, 12, 24 and 48 h.
Fragments of control genes were amplified by RT-PCR using total cellular RNA and appropriate primer pairs. Absolute expression of control genes was quantified based on a standard curve for commercial β-actin gene standard.

cell is not absolutely resistant to cell cycle fluctuations (Radonic *et al.*, 2004). Their variable expression may partially be explained by the fact that housekeeping proteins are not only implicated in the basal cell metabolism but also participate in other functions (Thellin *et al.*, 1999). β-Actin mRNA encodes a ubiquitous cytoskeleton protein and is expressed in almost all cell types. It was one of the first RNAs to be used as an internal standard, and is still advocated as a quantitative reference for RT-PCR assays (Bustin, 2000), although there is some evidence that its expression can be changed under various treatments (Giulietti *et al.*, 2001). The RNA encoding GAPDH is a ubiquitously expressed, moderately abundant message. GAPDH is not only a glycolytic enzyme, but it can also participate in different cellular processes (nuclear RNA export, DNA replication, cytoskeleton organization). Despite its prevalent use as a housekeeping gene, there is plenty of evidence that this gene is not a suitable endogenous control for quantification assays (Thellin *et al.*, 1999; Bustin 2000). In several recent articles the use of GAPDH as an endogenous control has been severely criticized because of the numerous situations where its expression is influenced by the experimental treatment or conditions (Giulietti *et al.*, 2001). Furthermore, it has been shown to be upregulated in cancer (Bustin, 2000).

Based on the analysis of the expression stability of both control genes in the present study and on the above experimentally documented facts, β-actin was chosen as a better reference gene for the study conditions because it exhibited only kinetic changes in the expression and greater stability in response to the treatment with different $InsP_6$ concentrations compared to GAPDH. The time fluctuations in β-

actin mRNA levels could be related to cell cycling since actin filaments' turnover is known to accompany cell division. It can be anticipated that the variability of β-actin expression observed in the HT-29 cells treated with different $InsP_6$ doses for prolonged time (48 h) could result from significant disturbances in cytoskeleton metabolism and architecture associated with the known growth inhibiting and proapoptotic influence of $InsP_6$ on cancer cells.

Evaluation of *p53* and *p21^WAF1* mRNAs level

Figure 5a–c shows the values of $2^{-\Delta\Delta Ct}$ reflecting the fold change in *p53* gene expression level in cells incubated with 1 mM (a), 5 mM (b), and 10 mM (c) $InsP_6$ for 6, 12, 24 and 48 h, calculated relative to the level of β-actin expression and the $\Delta\Delta Ct$ value ranges. The value of $2^{-\Delta\Delta Ct} > 1$ reflects increased ex-
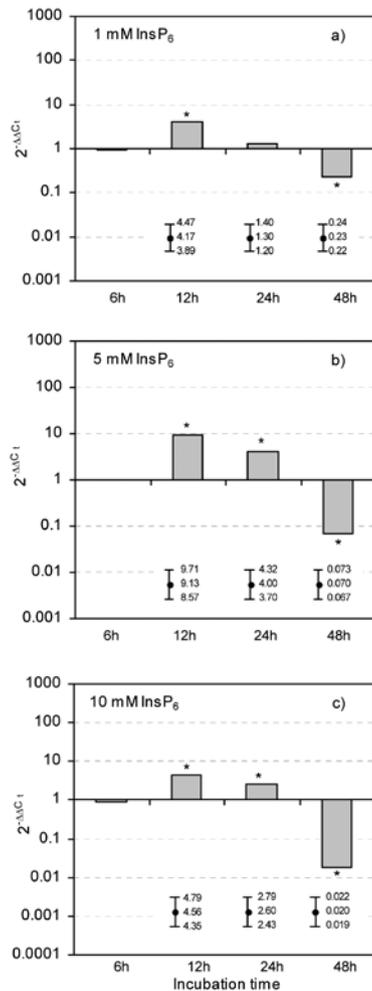

Figure 5. Changes in *p53* mRNA expression in HT-29 cells after treatment with $InsP_6$ for 6, 12, 24 and 48 h.
Detection and quantification of *p53* mRNA in total cellular RNA was performed by RT-PCR and ABI PRISM Sequence Detection System. The value of $2^{-\Delta\Delta Ct}$ represents the expression of the *p53* gene in $InsP_6$-treated cells normalized to β-actin relative to the normalized expression of *p53* gene in control cells. Mean values of $\Delta\Delta C_t$ and error range are also shown. * $P<0.05$ *vs* control cells.

pression of the target gene, and a value of $2^{-\Delta\Delta Ct} < 1$ points to a decrease in the gene expression. In all cell cultures treated with different doses of $InsP_6$ for 6, 12, 24 and 48 h, an increase in the expression of *p53* gene was detected at 12 h stimulation. The most effective concentration of $InsP_6$ appeared to be that of 5 mM, causing a 9-fold ($2^{-\Delta\Delta Ct}$ = 9.13) increase in the transcriptional activity of *p53* gene following 12 h treatment. At the two other concentrations, i.e., 1 and 10 mM with 12 h stimulation, $InsP_6$ up-regulated the *p53* expression to a similar extent, a 4.17- and 4.56-fold change, respectively. In cells incubated with 5 mM and 10 mM $InsP_6$, accumulation of *p53* mRNA at 24 h represented 4- and 2.6-fold increase of this gene's expression, respectively. At 48 h, in all cell cultures incubated with $InsP_6$ the relative *p53* gene expression revealed an opposite course. The observed effects of $InsP_6$ resulting in a decrease change in *p53* expression at 48 h (Fig. 5a–c) may be related to the very differentiated level of β-actin transcription in the presence of increasing concentrations of $InsP_6$ at 48 h (Fig. 4). The unstable transcription of the reference gene after 48 h of exposure of cells to $InsP_6$ suggests that the relative amount of *p53* transcript shown as a fold change in expression (Fig. 5a–c) reflects variation in both target and reference gene transcription caused by diverse cellular processes, including cell survival limitations under the longest treatment with $InsP_6$.

The fold change in the *p21^WAF1* gene transcription in cells treated with 1, 5, 10 mM $InsP_6$ for 6, 12, 24 and 48 h, normalized to β-actin, and the $\Delta\Delta Ct$ value ranges are presented in Fig. 6a–c. Accumulation of *p21^WAF1* transcript could be detected at 12 h of exposure to each of the $InsP_6$ concentrations, however, the highest positive changes in this gene's expression were monitored at 24 h incubation with 5 and 10 mM $InsP_6$. The most pronounced effect of $InsP_6$ was noted in cultures exposed to 5 mM $InsP_6$ at 24 h time point of incubation, resulting in a 150-fold increased *p21^WAF1* transcript level in the cells (Fig. 6b). The most significant increase in the transcriptional activity of *p21^WAF1* gene occurring at 24 h in response to 10 mM $InsP_6$ corresponded to a 10-fold increase in *p21^WAF1* mRNA (Fig. 6c). After 48 h incubation with 5 and 10 mM $InsP_6$, the *p21^WAF1* expression tended to decrease below the expression of this gene in the control cells not treated with $InsP_6$, as it was also observed for *p53* gene transcription at the same time point. After incubation of cells with 1 mM $InsP_6$, in contrast to the incubation with the two higher $InsP_6$ doses, the *p21^WAF1* transcription at 48 h was still detectable and it displayed a 2-fold increase over the control (Fig. 6a). It can be stated that the relative changes in *p21^WAF1* expression observed after 48 h, similarly to those in *p53* expression at 48 h, derived from two effects, one of them being the sig-
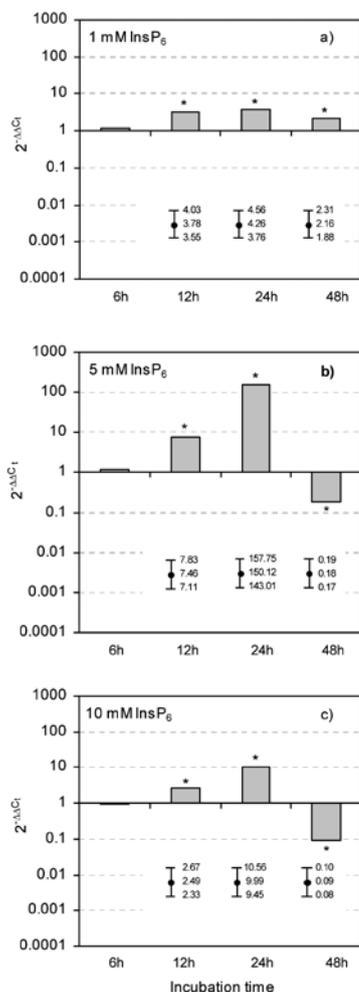
Figure 6. Changes in $p21^{WAF1}$ mRNA expression in HT-29 cells after treatment with $InsP_6$ for 6, 12, 24 and 48 h, in comparison with control cells.

Detection and quantification of $p21^{WAF1}$ mRNA in total cellular RNA was performed by RT-PCR and ABI PRISM Sequence Detection System. The value of $2^{-\Delta\Delta Ct}$ represents the expression of the $p21^{WAF1}$ gene in $InsP_6$-treated cells normalized to β-actin relative to the normalized expression of $p21^{WAF1}$ gene in control cells. Mean values of $\Delta\Delta C_t$ and error range are also shown. *$P < 0.05$ vs control cells.

nificant expression change of the reference gene in $InsP_6$-treated cells *versus* control cells at 48 h (Fig. 4). The magnitude of the changes in $p21^{WAF1}$ expression after 48 h in the presence of 5 and 10 mM $InsP_6$ was smaller compared to the changes in $p53$ expression at the corresponding doses, which suggests that the changes in the expression level of both target genes were different as they both were compared to the expression of the same reference gene, β-actin.

DISCUSSION

The chemopreventive effects of $InsP_6$ demonstrated against a variety of experimental tumors,

including colon cancer, still raise an important question about the molecular mechanisms of its growth inhibitory action. Mechanistic studies indicate that $InsP_6$ targets mitogenic and survival signaling in mammary and prostate cancer cells (Singh *et al.*, 2003; Vucenik *et al.*, 2005; Singh & Agarwal, 2005). Induction of $p21^{WAF1}$, a gene up-regulated by p53, is believed to be instrumental in cell growth inhibition. Therefore, the detection of $p21^{WAF1}$ induction may be related to p53 functional status and is useful for the analysis of p53-mediated cell-cycle checkpoints. The $p21^{WAF1}$ gene is also induced by p53-independent pathways (Chai *et al.*, 2000). Mutations of the $p53$ gene are present in many human tumors (Hainaut *et al.*, 1998). HT-29 cells have been shown to contain mutated $p53$ gene (Chen *et al.*, 2004). However, it has been found that in some cancer cell lines the $p53$ genomic changes did not overtly affect the expression of p53 protein because normal level of transcript and protein were found in these cells (Hsu *et al.*, 1993).

Immunocytochemical studies demonstrated increased levels of wild-type p53 and $p21^{WAF1}$ proteins in HT-29 cell line treated with $InsP_6$ in comparison with untreated cells (Saied & Shamsuddin, 1998). Studies by Vucenik *et al.* (1998) with the use of the same methods revealed that $InsP_6$ treatment of HepG2 human liver cancer cell line caused a decreased expression of mutant p53 protein with no significant change in the expression of wild-type p53. The authors hypothesized that this down-regulation of mutant p53 protein could be the result of either increased destruction or reduced synthesis due to possible activation of calpains by $InsP_6$ leading to a reduction of mutant p53 protein in mammalian cells (Vucenik *et al.*, 1998). This suggests that $InsP_6$ may act at the posttranslational or translational level, respectively. Furthermore, the p53 protein is known to be kept under cellular regulation, which in normal cells is critical considering its strong inhibitory activity on cell growth. The ability of p53 protein to adopt active and latent forms contributes to the regulation of its function (Kubbutat & Vousden, 1998). It has been indicated that the elevation of p53 protein levels in response to DNA damage occurs in the absence of clear changes in mRNA levels and that an increase in the protein level correlates with a prolonged half-life which indicates that protein stability might be important for controlling p53 function (Kubbutat & Vousden, 1998). The $p21^{WAF1}$ expression appears to be regulated at the posttranscriptional level too, because sometimes moderate increases in mRNA expression are followed by large increases in protein levels (Maltzman & Czyzyk, 1984). Based on these literature data, it can be concluded that the increased amounts of both proteins found in some cancer cells treated with $InsP_6$ cannot be a direct evidence of their induction at the gene level, but rather

again may suggest a role of InsP$_6$ in posttranscriptional regulation of protein amounts.

No studies have been done so far to analyze the effect of InsP$_6$ at the gene level in colon cancer epithelial cells by characterizing the changes in the dynamics of transcription of *p53* and *p21^WAF1* genes. Therefore, we analyzed quantitatively the level of *p53* and *p21^WAF1* transcripts produced in HT-29 cells as a function of time under treatment with different doses of InsP$_6$.

The results obtained in the present study showed that InsP$_6$ induced transcriptional activation of *p53* and *p21^WAF1* genes in HT-29 cells as reflected by the increase in their mRNA levels. This indicates that p21^WAF1 may be an effector of phytic acid-induced growth arrest in colon cancer cells. Litvak *et al.* (1998) found an increased level of p21 mRNA in Caco-2 cells, also having mutated *p53* gene, treated with sodium butyrate for 24 or 48 h, an agent known to halt proliferation of these cells. A transient increase in *p53* expression at the mRNA level was noted at 24 h. In an other kinetic study with the use of RT-PCR, of *p21* mRNA level after treatment of HT-29 cells with 5 mM sodium butyrate, this mRNA was detected after 6 h of incubation. Its transcription increased after 12 h and was stable at 24 h (Siavoshian *et al.*, 2000). In the studies by Fang *et al.* (2004), an RT-quantitative PCR assay was used to assess the transcription changes of *CDKN1A*, *p21^WAF1*, *p53*, and several other genes after treatment of human colon cancer cell lines Colo-320 and SW1116 with 5-aza-2'-deoxycytidine, trichostatin A (TSA) and sodium butyrate. Maximal enhancement of *p21^WAF1* expression was observed following TSA or sodium butyrate, with a 17–30-fold (Colo-320 cells) or 3–6-fold (SW1116 cells) increase in transcription.

In the present study, the maximum inducible effect of InsP$_6$ on *p21^WAF1* transcription in HT-29 cells was evident at 24 h and it occurred following the highest *p53* gene transcription observed at 12 h, indicating that *p21* may be induced by p53-dependent signal transduction. This conjecture is supported by the fact that an increase in wild type p53 protein was observed in these cells treated with InsP$_6$, as reported by Saied & Shamsuddin (1998). Taking into account the results of the present study showing the time relationship in activation of transcription of both genes along with the up-regulation of wild-type p53 protein in HT-29 cells (Saied & Shamsuddin, 1998) as well as down-regulation of mutant p53 protein in other cancer type cells in the presence of InsP$_6$ (Vucenik *et al.*, 1998), it can be concluded that in HT-29 cells InsP$_6$ stimulates a p53-dependent pathway of *p21^WAF1* gene induction. It is quite possible that InsP$_6$ may modulate the expression of p53 protein not only at the translational or posttranslational level by blocking the mutant p53 protein synthesis but also at the messenger RNA level, for instance by influencing a particular step of RNA maturation, such as primary transcript alternative splicing, leading to the appearance of the functional protein, an increased amount of which has been found in these cells following InsP$_6$ treatment.

The increase in the level of *p53* mRNA preceding that of *p21* mRNA, as observed in the present study, allows us to conclude that the up-regulation of *p21^WAF1* gene by InsP$_6$ in HT-29 colon cancer cells is mediated by a p53-dependent mechanism.

Acknowledgement

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