Epidemiological studies on the relationship between dietary habits and disease risk have shown that food has a direct impact on health (1). In recent years, natural plant products have attracted increased attention due to their beneficial effects on the prevention of various diseases such as cancer, cardiovascular, age-related and inflammatory diseases (2). Some natural agents may play a role in the alleviation and control of inflammation by influencing the production of proinflammatory cytokines and chemokines (3). Phytic acid (IP6) is one of the bioactive compounds found abundantly in cereals, legumes, oilseeds and nuts. It is also present in mammalian cells (4). IP6 has been recognized to possess various health benefits including anticancer effects (5, 6). Previous studies showed that it can also modulate immune functions of intestinal epithelium through regulation of the expression and secretion of interleukins such as IL-6 and IL-8 (7, 8), but mechanisms underlying that cellular response to IP6 have scarcely been examined.

Intestinal epithelial cells (IEC) produce cytokines and chemokines that play an important role in mucosal immune and inflammatory responses (9). A central pro-inflammatory chemokine involved in the pathogenesis of intestinal inflammatory diseases is IL-8 (10). IEC secrete IL-8 in response to cytokine stimulation (11), bacterial components such as lipopolysaccharide (11, 12), viral and bacterial infection (13). Furthermore, expression and secretion of IL-8 is elevated in various intestinal pathologies including inflammatory bowel diseases (IBD) and intestinal neoplasia (3, 14) and helps to sustain the ongoing inflammation (15). Modulation of the epithelial IL-8 production is very important in maintaining the intestine and body.
system in healthy condition and may constitute a key target in therapies for inflammatory disease of the intestine (10, 16). One of the signal transduction pathways involved in a variety of inflammatory responses, also in chemokine production, is p38 mitogen-activated protein kinase (MAPK) pathway (17). p38 MAP is a serine/threonine protein kinase activated by various extracellular stimuli that regulate the transcription of inflammatory cytokines, including IL-8 through a cascade of protein phosphorylation, leading to the activation of transcription factors (18).

In the present work, we investigated whether the in vitro influence of IP6 on IL-8 secretion by Caco-2 cells may be mediated by p38 MAP kinase. To address this issue, we analyzed the effect of IP6 on human p38α MAP kinase activity and the expression of gene encoding p38 MAP kinase in unstimulated and IL-1β-stimulated Caco-2 cells. Furthermore, the role of signaling pathways involving p38 MAP in IP6-induced down-regulation of IL-8 secretion by unstimulated and IL-1β-stimulated cells in the presence of p38 MAP kinase activator (anisomycin) and inhibitor (SB 203580) was evaluated.

EXPERIMENTAL

Reagents

Inositol hexakisphosphate as dipotassium salt and interleukin-1β were obtained from Sigma Aldrich. Human recombinant p38α MAP kinase was purchased from Cyclex MBL. Activators and inhibitors of p38 kinase: anisomycin, SB 205890 and SB 202190 were purchased from Sigma Aldrich.

Determination of the effect of IP6 on recombinant p38α MAPK activity

The activity of human recombinant p38α MAPK was measured by solid-phase enzyme immunoassay “Cyclex ® p38 Assay/Inhibitor Screening Kit” (Cyclex MBL) according to the manufacturer’s protocol. Enzyme activity was evaluated in the presence of various concentrations of IP6 (0.5, 1, 2, 2.5, 5 and 10 mM) and commercially available inhibitor of this enzyme SB202190. All assays of p38α MAPK in the control and test samples were performed in triplicate. The absorbance was measured at $\lambda = 450$ nm using the MRX Revelation microplate reader (DYNEX Technologies).

Cell culture

Caco-2 cells, a human intestinal epithelial cell line, were obtained from the American Type Culture Collection (ATCC). The cells were routinely grown in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; PAA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma Aldrich) and 10 mM HEPES (Sigma Aldrich). Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Cell culture for gene expression determination

To determine p38α MAP kinase gene expression, Caco-2 cells were seeded into 6-well tissue culture plates (Nunc) at an initial density of $4.5 \times 10^5$ cells/well in 3 mL of RPMI 1640 medium supplemented with the components given above. Three days after plating, the culture medium was changed to RPMI 1640 containing 2% FBS and the cells were cultured for 24 h. Afterwards, cells were treated with 1 mM IP6 for 3, 6 and 12 h. Parallel sets of
cultures were treated with 1 ng/mL IL-1β for 30 min prior to IP6 adding. The IP6 untreated cells were used as a control.

RNA extraction

Total cellular RNA was extracted from control and treated with IL-1β and IP6 cells by using TRI- ZOL® reagent (Invitrogen) according to the manufacturer’s protocol. RNA quality was assessed by 1.0% agarose gel electrophoresis in the presence of ethidium bromide. The RNA concentration was determined spectrophotometrically using a GeneQuant pro (Amersham Biosciences).

Real-time RT-PCR assay

Transcriptional activity of gene coding for p38α MAP kinase was evaluated by the use of real time RT-PCR technique (with TaqMan probes). p38α MAP kinase gene expression profiles were determined using a commercially available set of oligonucleotide primers and probe (Hs00176247_m1, Applied Biosystems). The analyses were carried out using an Opticon™ DNA Engine detector (MJ Research, USA). The thermal profile of one-step RT-PCR was as follows: 50°C for 30 min (reverse transcription) followed by 95°C for 15 min (DNA polymerase activation) and then 45 cycles of amplification at 94°C for 15 s and 60°C for 1 min each. QRT-PCR assay was performed in triplicate for each sample. The obtained results of mRNA copy number were recalculated per µg of total RNA. Finally, specificity of RT-PCR reaction was confirmed by 6% polyacrylamide electrophoresis of RT-PCR products with their visualization using silver staining.

Figure 2. Comparison of kinase MAP p38α gene expression in Caco-2 cells unstimulated (A) and IL-1β-stimulated (B) treated with 1 mM IP6 for 3, 6 and 12 h (the mean ± SD; * p < 0.05 vs. IP6-untreated cells)
Cell culture treatment for interleukin-8 secretion determination

Caco-2 cells were seeded into 24-well tissue culture plates (Nunc) at an initial density of $1 \times 10^5$ cells/well in 1 mL RPMI 1640 medium supplemented with the components given above. Three days after plating, the culture media were changed to media with 2% FBS and cells were then cultured for 2 days. Afterwards, the media were replaced with fresh ones containing 2% FBS and cells were incubated for 30 min with either 500 ng/mL µM anisomycin (an activator of p38 MAPK) or 10 µM SB 205890 (an inhibitor of p38 MAPK) before IP6 (1 and 2.5 mM) treatment. Parallel sets of cultures were treated with 1 ng/mL IL-1β and either anisomycin (500 ng/mL) or SB 205890 (10 µM) for 30 min prior to IP6 adding. Separate cultures were incubated with IP6 only and were preincubated with 1 ng/mL IL-1β for 30 min before IP6 treatment. After 24 h, the media were collected for IL-8 assay.

Each control and treatment group was performed in triplicate. Cell monolayers were lysed for 5 min with 0.1% sodium dodecyl sulfate (SDS, Sigma Aldrich). After centrifugation, protein concentration was assayed in the supernatants using the Bradford reagent (Sigma Aldrich) according to the manufacturer’s protocol. Bovine serum albumin was used as a standard. The absorbance was measured at a wavelength of 595 nm using the MRX Revelation microplate reader (DYNEX Technologies).

Measurement of interleukin-8

The amounts of IL-8 secreted into the culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine R&D Systems) following the manufacturer’s protocol. All assays of IL-8 concentration were performed in triplicate. The absorbance was measured using the MRX Revelation plate reader (DYNEX Technologies) at $\lambda = 450$ nm. The concentrations of...
IL-8 were normalized to the total protein content in the cells and expressed as picograms per milligram of protein.

**Statistical analysis**

All results were expressed as the means ± standard deviation (SD). Statistical analysis was performed with the use of Statistica PL ver. 9.0 Software (StatSoft). Student’s *t*-test was used to assess statistical significance of difference between two groups. For multiple comparisons one-way analysis of variance (ANOVA) was performed followed by post-hoc Tukey’s test. Differences were considered statistically significant when the probability value *p* was lower than 0.05.

**RESULTS**

**The effect of IP6 on human recombinant p38MAP kinase activity**

The influence of IP6 (0.5; 1; 2.5; 5 and 10 mM) on the activity of human recombinant p38MAP kinase in comparison to that exerted by p38 kinase inhibitor SB 202190 (10 μM) is shown in Figure 1. Incubation with SB 202190 resulted in almost complete (> 98%) reduction of kinase activity. IP6 reduced its activity in a concentration-dependent manner. A half mM IP6 decreased kinase activity by 5.8%, but observed effect was not statistically significant (*p* > 0.05). Statistically significant inhibition of enzyme activity was achieved with higher concentrations of IP6 (≥ 1 mM). 1 and 2 mM IP6 caused 29.97% and 63.3% inhibition of kinase activity, respectively, and this effect was weaker than that of the inhibitor SB 202190 (*p* > 0.05). Treatment with IP6 at 2.5, 5 and 10 mM led to further inhibition of enzyme activity (by 85.25, 99.38 and 98.66%, respectively) and statistical analysis revealed no significant differences between the effect of evoked by IP6 and SB 202190.

**Changes in expression of p38MAP kinase gene in unstimulated and IL-1β-stimulated Caco-2 cells treated with IP6**

The p38αmRNA level in unstimulated and IL-1β-stimulated Caco-2 cells treated with IP6 was determined using real-time RT-PCR assay. Both untreated cells and cells treated with 1 mM IP6 for 3, 6 and 12 h showed the expression of p38αmRNA and the transcript level of p38α at 3 h demonstrated statistically significant differences (Fig. 2A). The constitutive expression of p38αmRNA was down-regulated in cells by 1 mM IP6 (*p* = 0.041) as compared to the control (Fig. 2A). The similar level of p38αmRNA was found in untreated and treated with IP6 cells after 6 and 12 h (*p > 0.05*) (Fig. 2A).

In IL-1β-stimulated Caco-2 cells the level of p38α transcript was significantly lowered by 1 mM IP6 (*p* = 0.02) at 3 h in comparison with IP6 untreated cells (Fig. 2B). No statistically significant changes in the IL-8 gene expression in the cells treated with IP6 for 6 and 12 h compared to the control cells have been found (*p > 0.05*) (Fig. 2B).

**The influence of IP6, anisomycin and SB 203580 on constitutive secretion of IL-8 by Caco-2 cells**

A multiplicity of intracellular signal-transduction pathways can be involved in the regulation of IL-8 secretion. To assess the role of signaling pathways involving p38 kinase MAP in IL-8 release by Caco-2 cells, anisomycin – a p38 MAP kinase activator and SB 203580 – a p38 MAP kinase inhibitor were used. Incubation of cells with anisomycin resulted in an 11.8-fold up-regulation of IL-8 secretion (*p < 0.001*). SB 203580 had a suppressive effect on constitutive IL-8 release by cells, nevertheless with no significant differences in relation to control (*p > 0.05*) (Fig. 3A).

To define whether IP6 can modify the release of IL-8 by influencing signaling pathway involving p38 MAP kinase, Caco-2 cells were preincubated with anisomycin and SB 203590 prior to IP6 treatment. A 8.2-fold increase in IL-8 secretion (*p < 0.001*) and not significant differences in IL-8 amounts were detected in cultures preincubated with p38 kinase inhibitor in comparison with cells treated with IP6 only (Fig. 3B).

Furthermore, as shown in Figure 3, IP6 decreased constitutive secretion of IL-8 by 30% (*p = 0.006*) and down-regulated IL-8 release evoked by both compounds modulating p38 MAPK activity. A 51% reduction in stimulated by p38 MAPK activator IL-8 secretion (*p < 0.0001* vs. cells treated with anisomycin alone) (Fig. 3) as well as a 19.57% decrease in IL-8 in cultures preincubated with SB 203580 (in comparison with cells incubated with inhibitor alone, *p = 0.025*) (Fig. 3) could be observed following IP6 use.

**Changes in IL-1β stimulated secretion of IL-8 by Caco-2 cells treated with IP6 in the presence of p38 MAP kinase activator and inhibitor**

Furthermore, the effect of anisomycin and SB 203580 on IL-8 release by Caco-2 cells stimulated with IL-1β was evaluated (Fig. 4A). Incubation of these cells with anisomycin resulted in 6.5-fold up regulation of IL-8 secretion (*p < 0.001*) and their
treatment with p38-specific inhibitor showed ~ 30% reduction in IL-8 secretion (p < 0.005).

The effect of IP6 on IL-8 secretion by IL-1β-induced Caco-2 cells preincubated with compounds modulating p38 MAPK activity is shown in Figure 4B. Incubation of stimulated by IL-1β cells with anisomycin and IP6 caused a 10-fold increase in IL-8 secretion vs. cultures without anisomycin (p < 0.001). Moreover, IL-8 secretion was almost completely inhibited (84%) by IP6 in IL-1β-stimulated cells preincubated with SB203580 in relation to IP6-treated cultures with no inhibitor (p < 0.001) (Fig. 4B).

As shown in Figure 4, IL-8 secretion in IL-1β-stimulated cells was markedly inhibited (48%) by IP6 (p < 0.005). Also, in IL-1β-stimulated cells preincubated with anisomycin, IP6 down-regulated secretion of IL-8 by 17% (p < 0.001 vs. cells treated with anisomycin alone), however, the inhibitory effect of IP6 was weaker than in cultures without anisomycin. IP6 promoted 87% decrease in IL-8 release by IL-1β-stimulated cells preincubated with SB203580 as compared to cells treated with inhibitor only (p < 0.0001).

**DISCUSSION and CONCLUSION**

Recent trends in prevention and treatment of intestinal inflammation are focused on finding natural compounds that inhibit inflammatory reaction. Dietary components can modulate the inflammatory response of IEC by regulating the expression and secretion of cytokines. The modulating roles of nutrients in IL-8 secretion from IEC have recently received increasing attention (16). Previous study has shown that a natural dietary component ñ IP6 exerts its biological activity via influencing protein kinases. Some studies recognized protein kinases like PI3K (28), PKC (29) and MAPK (30) as molecular targets of IP6. Studies by Jagadeesh and Banerjee (19) showed that IP6 inhibited phosphorylation of Akt and PKC ßin prostate cancer cells. Gu and coworkers (31) have reported that IP6 decreased the levels of phosphorylated ERK1/2, JNK1/2 and p38 in human prostate carcinoma PC-3 cells. These observations parallel with ours in that we also observed that IP6 exerts its biological activity via influencing protein kinases.

The expression and secretion of IL-8 is subject to complex intracellular control, which involves multiple signaling pathways such as the MAPK pathway (19). These cascade mediate cellular response to external stress signals, which make them potential targets for anti-inflammatory therapies. p38 MAP kinase is an important signal molecule involved in the expression and secretion of inflammatory mediators. There is increasing evidence that p38 signaling pathways play a role in intestinal inflammation (20, 21) and p38 MAP kinase has been reported to be the most important isoform of this enzyme involved in the intestinal inflammatory response (22, 23). Thus, the present study evaluated whether inhibitory effect of IP6 on IL-8 secretion by Caco-2 cells could be mediated by p38 MAP kinase. In this experiment, the effect of IP6 on human recombinant p38α MAP kinase activity was studied. The results have shown that activity of this enzyme was down-regulated by IP6 in a dose-dependent manner. This study has also shown that IP6 inhibits expression of p38α MAP kinase gene in Caco-2 cells both unstimulated and stimulated with IL-1β, which is a key regulator of epithelial function during inflammation. Furthermore, van de Walle et al. (11) demonstrated that IL-1β is a major determinant of the production of IL-8 in Caco-2 intestinal epithelial cells.

Several studies have suggested that the signaling pathways involving p38 MAP kinase played an important role in the regulation of IL-8 secretion by various cell types (10, 24, 25). The aim of this experiment was to evaluate whether these signaling pathways play a role in IL-8 secretion by Caco-2 cells. It has been shown that activation of this enzyme strongly stimulated IL-8 secretion and SB203580 inhibited induction of IL-8 by IL-1β. These data indicate that p38 MAP kinase is involved in secretion of IL-8 by Caco-2 cells. The role of this enzyme in stimulated IL-8 secretion by intestinal epithelial cell lines was also confirmed by other studies (26, 27).

Furthermore, in the current study, the effect of IP6 on p38 MAPK dependent IL-8 secretion by unstimulated and IL-1β-stimulated Caco-2 cells was evaluated. In cells preincubated with anisomycin, IP6 caused a decrease of IL-8 secretion stimulated with anisomycin and IL-1β. These findings suggest that down-regulatory effect of IP6 on IL-8 release could involve inhibition of p38 MAPK signaling pathways. Preincubation with SB203580 before IP6 treatment augmented further reduction in IL-8 secretion by both unstimulated and stimulated with IL-1β cultures compared to cells treated with SB203580 alone. It seems that signaling pathways other than p38 MAPK are likely to participate in secretion of IL-8 by Caco-2 cells treated with IP6.
In conclusion, the present results show that physiological intestinal concentrations of IP6 may have an inhibitory effect on IL-8 secretion and one of the mechanisms of its action is down-regulation of p38 MAP kinase signaling cascade. The results of these studies suggest that dietary IP6 could be a good candidate for both prevention and treatments of inflammatory events in the intestinal epithelium.

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