Intestinal epithelial cells (IEC) form a physical barrier between the contents of the gut lumen and the underlying tissues. In addition, these cells appear to function as an integral component of the mucosal immune system. IEC may not only serve as targets for locally produced inflammatory mediators such as cytokines but also participate in inflammatory or immune responses in gut by producing and secreting cytokines and chemokines (1). A lot of evidences indicate that a dysregulation of mucosal immunity in the gut as observed in inflammatory bowel disease (IBD) causes an overproduction of inflammatory cytokines leading to uncontrolled intestinal inflammation and disruption of epithelial function (2).

IL-8, a member of chemokine family, has been recognized to play an important but unspecific role in the pathogenesis of inflammatory diseases of the intestines. The production of IL-8 by IEC is promoted by various stimuli like bacteria, bacterial lipopolysaccharides, proinflammatory cytokines such as IL-1β, TNF-α and oxidative stress (3–6). As a potent chemoattractant and activating cytokine for neutrophils and other leukocytes, IL-8 participates in the recruitment and infiltration of these cells in intestinal mucosa (5). Elevated levels of IL-8 have been found in the colonic mucosa of people suffering from ulcerative colitis and Crohn disease (7). Increased IL-8 expression is observed in both acute and chronic inflammation (8). Thus, modulating IL-8 secretion may constitute a key target of therapy for chronic inflammatory diseases and related cancers in the gastrointestinal tract.

Some nutrient constituents could contribute to the modulation of gut inflammation and cytokine secretion. Among dietary agents, phytic acid (inositol hexakisphosphate, IP6) has been the focus of interest in the latest years due to its chemopreventive properties against various types of cancer including colorectal cancer (9, 10). IP6 is a naturally occurring phytochemical abundant in many plants and various high-fiber foods such as whole grains, cereals, legumes, soy, oil seeds and nuts (9, 11–13).

ROLE OF PROTEIN TYROSINE KINASE IN THE EFFECT OF IP6 ON IL-8 SECRETION IN INTESTINAL EPITHELIAL CELLS

JOANNA WAWSZCZYK*, ARKADIUSZ ORCHEL, MALGORZATA KAPRAL and LUDMILA WĘGLARZ

1Department of Biochemistry, 2Department of Biopharmacy, Medical University of Silesia, Narcyzów 1, 41-200 Sosnowiec, Poland

Abstract: Phytic acid (IP6) is a major fiber-associated component of a diet physiologically present in human intestines. Studies showed that this phytochemical can modulate immune functions of intestinal epithelium through regulation of proinflammatory cytokines secretion but mechanisms underlying these cellular response to IP6 have weakly been examined, as yet. The aim of this study was to determine the role of protein tyrosine kinase (PTK) in secretion of IL-8, a central proinflammatory cytokine, by unstimulated and IL-1β-stimulated intestinal epithelial cells Caco-2 treated with IP6 (1 and 2.5 mM). To study the involvement of PTK signal pathway in IL-8 secretion, inhibitors of phosphotyrosine phosphatase (sodium orthovanadate, OV) and tyrosine kinase (genistein, GEN) were incubated with Caco-2 cells prior to IP6 treatment. IP6 had suppressive effect on basal and IL-1β-stimulated IL-8 secretion by cells. The effect of OV on IL-8 release by cells treated with IP6 was different under constitutive and stimulated conditions. Secretion of IL-8 was significantly down-regulated in cells with GEN and GEN plus IP6 treatment. In addition, total PTK activity in both unstimulated and IL-1β-stimulated cells was determined in the presence of IP6. The results suggest that physiological intestinal concentrations of IP6 may have an inhibitory effect on IL-8 secretion by Caco-2 cells and one of the mechanisms of its action is the inhibition of PTK signaling cascade. The study revealed for the first time that PTKs could be one of the molecular targets for IP6 effects in the intestinal epithelial cells.

Keywords: phytic acid, IL-8, intestinal epithelial cells Caco-2, IL-1β, protein tyrosine kinase

* Corresponding author: e-mail: jwawszczyk@sum.edu.pl
It is also found in most mammalian cells at levels ranging from 10 µM to 1 mM (14). High luminal concentrations of IP6 reaching 4 mM are present physiologically in human large gut (15). Several studies documented the ability of IP6 not only to reduce the risk of developing cancer but also inhibit tumor progression in animal cancer models (14). In addition to its anticancer potential, numerous activities of IP6 beneficial for human health have been reported, such as reduction of serum lipids levels (16), prevention of pathological calcification and kidney stone formation (17), inhibition of platelet activation (18), protection of myocardium from ischemic damage and reperfusion injury (19) and antioxidative action. Moreover, IP6 has been shown to have an impact on the inflammatory response involving regulation of immune cell activity. It enhances natural killer cell activity in vitro (14), functions as a neutrophil priming agent (20) and augments host defense via stimulation of respiratory burst in stimulated human polymorphonuclear leukocytes (21). The immunoregulatory role of IP6 has also been demonstrated by our previous and others’ studies showing that IP6 could modulate the expression and secretion of cytokines IL-8, IL-6, TNF-α, TGF-β by epithelial intestinal cells and peripheral blood mononuclear cells in response to stimuli such as bacterial LPS and IL-1β (22–24). It has been shown that the regulation of expression and secretion of cytokines by cells is associated with different intracellular signal transduction pathways inter alia mitogen-activated protein kinases (MAPK), protein kinase A (PKA), protein kinase C (PKC) and those involving protein tyrosine kinases (PTK) (25, 26).

Since IEC produce IL-8 and increase its secretion in response to IL-1β, the study of the role of protein kinases in the induction of IL-8 secretion in human intestinal epithelial cells (Caco-2) was undertaken in order to get insight into the signal transduction pathways utilized in IL-8 secretion in these cells. To investigate whether the modulatory mechanism of IL-8 secretion by IP6 involves the regulation of protein tyrosine kinases, we analyzed the role of PTK signaling pathways in Caco-2 cells by using phosphotyrosine phosphatase inhibitor (sodium orthovanadate, OV) and an inhibitor of protein tyrosine kinase (genistein, GEN). In addition, in the experiment, total protein tyrosine kinase activity in both unstimulated and IL-1β-stimulated Caco-2 cells was determined following their treatment with IP6.

EXPERIMENTAL

Reagents

Inositol hexakisphosphate and interleukin-1β was obtained from Sigma-Aldrich. Sodium orthovanadate (a protein phosphotyrosine phosphatase inhibitor) and genistein (a protein tyrosine kinase inhibitor) were purchased from Calbiochem.

Cell culture

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

Cell culture treatment for interleukin-8 secretion determination

To determine IL-8 secretion, Caco-2 cells were seeded into 24-well tissue culture plates (Nunc) at an initial density of 1 × 10⁵ cells/well in 1 ml RPMI...
Effect of phytic acid on constitutive and IL-1β-stimulated secretion of IL-8 by Caco-2 cells

Caco-2 cells constitutively released IL-8 at a mean amount of 636 ± 55 pg/mg. Secretion of IL-8 was significantly up-regulated upon activation of cells by IL-1β (13 905 ± 455 pg/mg protein) (p < 0.001). Changes in IL-8 secretion by unstimulated
and stimulated with IL-1β Caco-2 cells evoked by IP6 (1 and 2.5 mM) expressed as percent of the corresponding control value are shown in Fig. 1A and 1B, respectively. IP6 had a statistically significant, suppressive effect on constitutive IL-8 release by cells leading to its 39% and 41.5% decrease depending on the concentration (1 and 2.5 mM IP6, respectively) in relation to control. No significant difference was observed in concentration of IL-8 between cultures treated with 1 and 2.5 mM IP6. IP6 had also a suppressive, dose-dependent influence on IL-8 release by cells stimulated with IL-1β, i.e., 9% (1 mM) and 21% (2.5 mM), respectively. The down-regulatory effect of IP6 on IL-8 release by stimulated Caco-2 cells was statistically significant (p < 0.05). The differences between inhibitory effect of 1 and 2.5 mM IP6 on IL-8 secretion by IL-1β-stimulated cells were statistically significant (p < 0.05).

Role of protein tyrosine kinases in constitutive and IL-1β-stimulated IL-8 secretion by Caco-2 cells

To evaluate the role of intracellular signaling pathway involving protein tyrosine kinases in the regulation of IL-8 secretion by Caco-2 cells, sodium orthovanadate (OV) – a protein phosphotyrosine phosphatase inhibitor and genistein (GEN) – protein tyrosine kinase inhibitor were used. As shown in Fig. 2A, incubation of cells with OV did not influence IL-8 amount released following cells culturing in the absence of IL-1β (p > 0.05). However, when IL-1β-stimulated cells were incubated with OV, 1.3-fold increase of IL-8 secretion (p = 0.0019) was observed (Fig. 2B). Incubation of cells with genistein resulted in significant inhibition of IL-8 secretion both in absence and presence of IL-1β, i.e., by 85.5% and 86%, respectively (p < 0.001).

Figure 2. Effect of sodium orthovanadate and genistein on IL-8 secretion by unstimulated (A) and stimulated with IL-1β (B) Caco-2 cells. The results are expressed as the means ± SD. *p < 0.05 vs. control

Figure 3. Effect of (A) IP6, (B) sodium orthovanadate and IP6 and (C) genistein and IP6 on IL-8 release by Caco-2 cells. Results are expressed in percents of basal IL-8 secretion (control). Values are the means ± SD, *p < 0.05
Effect of phytic acid in the presence of tyrosine kinase and phosphatase inhibitors on constitutive secretion of IL-8 by Caco-2 cells

A multiplicity of cellular signaling pathways can be involved in the regulation of IL-8 secretion. To define whether phytic acid can regulate the production of IL-8 by influencing signaling pathway involving protein tyrosine kinases, Caco-2 cells were preincubated with sodium orthovanadate and genistein before IP6 treatment. The data from these experiments are summarized in Fig. 3. IP6 modified the response of cells treated with OV (Fig. 3B). The significantly down-regulatory effect of IP6 on the IL-8 secretion was weaker in cells preincubated with OV compared to its effect in cells cultured without OV (Fig. 3B vs. Fig. 3A). IP6 caused a 39% and 41% decrease in IL-8 concentration in cell culture supernatants (Fig. 3A) in relation to untreated cultures (Control) and it provoked only 20% and 26% decrease in IL-8 amounts in culture supernatants following treatment with OV compared to cells treated with OV only (Fig. 3B). Incubation of Caco-2 cells with genistein or genistein plus IP6 resulted in significant inhibition of IL-8 secretion (Fig. 3C). There were no significant differences between IL-8 levels in culture media collected from cells treated with GEN alone and cells incubated with GEN plus IP6.

The effect of phytic acid in the presence of tyrosine kinase and phosphatase inhibitors on IL-1β stimulated secretion of IL-8 by Caco-2 cells

Furthermore, also the effect of OV and GEN on IL-8 secretion by cells stimulated with IL-1β was evaluated. The IL-8 release in response to IL-1β stimulation was significantly inhibited by IP6 (Fig. 4A). Pretreatment with OV of IL-1β-stimulated Caco-2 cells attenuated an inhibitory effect of IP6 on IL-8 secretion (Fig. 4B vs. Fig. 4A). IP6 at concentrations of 1 and 2.5 mM promoted 11% decrease in IL-8 release by IL-1β-stimulated cells incubated with OV compared to cells treated with OV only. However, the down-regulatory effect of IP6 on the IL-8 secretion by cells incubated with OV was not found to be statistically significant compared to cells treated with OV alone (Fig. 4B). Incubation of stimulated by IL-1β Caco-2 cells with genistein alone or genistein and IP6 caused a strong inhibition of IL-8 secretion (Fig. 4C). There were no significant differences between IL-8 levels in culture media collected from cells treated with GEN alone and cells incubated with GEN plus IP6.

Effect of phytic acid on tyrosine kinase activity in Caco-2 cells not stimulated and stimulated with IL-1β

To further examine whether tyrosine kinases activity could be changed with IP6 treatment, total tyrosine kinases activity in unstimulated and IL-1β-stimulated colonocytes treated with IP6 was evaluated in cell-free lysates (Fig. 5). The results indicate that incubation of cells with IL-1β caused statistically significant increase of PTK activity compared to control cells (p = 0.018). As shown in Fig. 5A, treatment of unstimulated cells with 1mM and 2.5 mM IP6 decreased PTK activity (p = 0.017 and p < 0.001, respectively). No significant differences in PTK activity between cultures treated with IP6 at 1
and 2.5 mM doses were observed. Furthermore, the data presented in Fig. 5B indicate that IP6 at concentrations of 1 and 2.5 mM caused essential down-regulation of PTK activity also in cells stimulated with IL-1β (p < 0.001) and the observed reduction of PTK activity was greater than in unstimulated cells. No significant differences in PTK activity between IL-1β-stimulated cells treated with 1 mM and 2.5 mM IP6 were observed.

DISCUSSION AND CONCLUSIONS

Epidemiological studies have clearly indicated an inverse association between dietary fibre and whole-grain cereal consumption and intestinal cancer incidence (12). Furthermore, it was noticed that only fiber with high IP6 content, such as cereals and legumes, show negative correlation with colon cancer (26). This anti-carcinogenic effect may be attributed to the antioxidant and anti-inflammatory properties of bioactive components of fiber such as IP6, as increased oxidative stress and inflammation are involved in cancer etiology (27). Patients with long-standing IBD have an increased risk of developing colorectal cancer. Therefore, recent trends of prevention and treatment of IBD have focused on searching for compounds which specifically inhibit the molecules involved in the inflammatory cascade (28). One of the major targets for such treatment are cytokines and their receptors produced by IEC (29, 30). Increasing evidence suggests that dietary factors can modulate different immune functions of epithelial cells lining of the gastrointestinal tract (12, 31). It has been reported that dietary phenolic compounds may diminish the production of proinflammatory cytokines by intestinal epithelial cells (32). Also long-chain dietary fatty acids modulate interferon production by intraepithelial lymphocytes (33).

Our previous and the present studies have demonstrated the ability of IP6 to down-regulate of proinflammatory cytokines secretion by intestinal epithelial cells Caco-2 (22). This cell line represents a well-established and widely used model of the human intestinal epithelium (34). Caco-2 cells were exposed to pro-inflammatory stimulus IL-1β in order to mimic an inflamed intestinal epithelium. IL-1β shares a multitude of proinflammatory properties and appears to be critical to the amplification of mucosal inflammation (2). Enhanced expression of IL-1β in the intestinal tissue has been found in IBD patients (2, 35). IL-1β is secreted by monocytes and macrophages and induces IEC to release inflammatory mediators like IL-8 (2, 22).

This study has shown that IP6 used at realistic intestinal concentration modulates the immunologic function of IEC by decreasing constitutive and IL-1β-stimulated IL-8 secretion. Such an inhibitory effect of IP6 would effectively serve to suppress inflammatory response in the intestinal epithelium. The expression and secretion of IL-8 is subject to complex intracellular control, which involves signaling pathways transmitting signals to the nucleus through protein kinases (36). Several studies have suggested that the signaling pathways involving protein tyrosine kinases played an important role in the regulation of IL-8 secretion by various cell types (2, 37). Thus, the aim of this study was to evaluate whether these signaling pathways play a role in IL-8 secretion by Caco-2 cells. To investigate the role of PTK in IL-8 secretion, cells were treated with OV and GEN. OV is an inhibitor of protein phosphotyrosine phosphatase that can up-regulate tyrosine kinase-linked signaling through the inhibition of tyrosine residue dephosphorylation. GEN is a broad-spectrum, highly specific tyrosine kinase inhibitor that blocks both receptor and non-receptor protein tyrosine kinases (36). OV did
not induced the secretion of IL-8 by unstimulated cells, but in case of cells stimulated with IL-1β it led to a statistically significant increase in secretion of IL-8 in relation to the control culture. The IL-8 secretion was greatly inhibited by GEN both in unstimulated and stimulated with IL-1β cells, which indicates that tyrosine kinases are involved in both constitutive and IL-1β-stimulated secretion of IL-8 by Caco-2 cells. The role of protein tyrosine kinases in stimulated IL-8 secretion by intestinal epithelial cell line HT-29 was confirmed by other studies. Genistein and herbimycin almost completely reduced IL-8 secretion induced by IL-1β, TNF-α and bacterial enterotoxin in HT-29 cells (28, 36). The role of tyrosine kinases in IL-8 secretion has also been confirmed in human endothelial cells (38) and the retinal pigment epithelial cells stimulated with glycated albumin (37).

Furthermore, in the current study, the effect of IP6 on PTKs dependent IL-8 secretion by unstimulated and IL-1β-stimulated Caco-2 cells was evaluated. Preincubation of cells with OV resulted in a weaker inhibition of IL-8 secretion by cells incubated with IP6. No significant differences were observed in the amounts of IL-8 between cells incubated with IP6 plus GEN and those treated with GEN alone. This observation indicates that IL-8 secretion by Caco-2 cells depends on tyrosine phosphorylation and that IP6 influences IL-8 secretion by affecting enzymes involved in signal transduction pathways involving PTKs.

In IL-1β-stimulated cells preincubated with OV, IP6 caused a decrease of IL-8 secretion, but this effect was not statistically significant. In the presence of OV a weaker inhibitory effect of IP6 on IL-8 release could be explained by its opposite effect on the processes of protein phosphorylation. Sodium orthovanadate eliminates the effects caused by the presence of phytic acid. In turn, when there was no phytic acid present, OV enhanced the effect of IL-1β because it blocked the inactivation of signaling pathways activated by IL-1β (phosphate cleavage from activated protein was inhibited). As in the case of unstimulated cells, preincubation of IL-1β-stimulated cells with GEN before IP6 treatment did not augment further reduction in IL-8 secretion compared to cells treated with genistein alone. These findings suggest that IL-8 secretion by IL-1β-stimulated colonocytes is dependent on PTKs activity and that down-regulatory effect of IP6 on IL-8 release could involve inhibition of PTK signaling.

A few reports documented protein kinases as molecular targets of phytic acid. IP6 affected such protein kinases as phosphatidylinositol-3 kinase (39, 40), PKC (41, 42) or MAPK (39, 41, 43). Studies by Gu et al. (44) showed that IP6 not only decreased the levels of phosphorylated ERK1/2, JNK1/2 and p38 in human prostate carcinoma PC-3 cells but also could be effective in blunting both pro-survival and mitogenic signaling in prostate cancer cells. In the present study total PTK activity in not stimulated and stimulated with IL1-β cells treated with IP6 was measured. Phosphorylation of tyrosine residues on regulatory proteins by PTKs occurs at multiple steps in the signaling cascades that regulate the expression and production of proinflammatory mediators. The enhanced PTKs activity has been implicated in the pathophysiology of diseases associated with inflammation including that in IBD (45).

Stimulation of Caco-2 cells with IL-1β caused significant increase of PTK activity. Gross et al. (6) reported that incubation of HT-29 cells with IL-1β or TNF-α did not cause essential changes in total PTK activity. The authors concluded that the effects evoked by stimulation of cells with IL-1β or TNF-α might be explained by changes in the intracellular localization of PTKs. In the present study, it has also been found that IP6 treatment resulted in an inhibition of PTK activity in Caco-2 cells both unstimulated and IL-1β-stimulated. These results revealed for the first time that one of the molecular targets for IP6 in intestinal epithelial cells Caco-2 are tyrosine kinases. The other molecular targets of this bioactive compound are still to be identified.

It is evident from the results reported here that the potent inhibition of PTK activity by IP6 does not result in almost complete inhibition of IL-8 secretion. It seems that signaling pathways dependent on phosphorylation of proteins other than protein tyrosine kinases 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 protein tyrosine kinase signaling cascade. The results of these studies suggest that IP6 could be one of the naturally occurring compounds that normally regulates immune reactivity at the mucosal surface by a direct effect on the intracellular events involved in cytokine release in intestinal epithelial cells. Thus, diets rich in IP6, such as those high in certain dietary fibers, could be beneficial not only for preventing human malignancies in the colon, but also for preventing or limiting inflammatory events in the intestinal epithelium.
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