Intestinal alkaline phosphatase inhibits the proinflammatory nucleotide uridine diphosphate

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ABSTRACT

Uridine diphosphate (UDP) is a proinflammatory nucleotide implicated in inflammatory bowel disease. Intestinal alkaline phosphatase (IAP) is a gut mucosal defense factor capable of inhibiting intestinal inflammation. We used the malachite green assay to show that IAP dephosphorylates UDP. To study the anti-inflammatory effect of IAP, UDP or other proinflammatory ligands (LPS, flagellin, Pam3Cys, and TNF-α) in the presence or absence of IAP were applied to cell cultures, and interleukin-8 was measured. UDP caused dose-dependent increase in IL-8 by both immune cells and two gut epithelial cell lines, and IAP treatment abrogated IL-8 release. Co-stimulation with UDP and other inflammatory ligands resulted in a synergistic increase in IL-8 release, which IAP treatment prevented. In vivo, UDP +/- IAP was instilled into a small intestinal loop model in wild-type and IAP-knockout mice. Luminal contents were applied to cell culture, and cytokine levels were measured in both culture supernatant and intestinal tissue. UDP-treated luminal contents induced more inflammation on target cells, with a greater inflammatory response due to contents from IAP-KO mice treated with UDP compared to WT. Additionally, UDP treatment increased TNF-α levels in intestinal tissue of IAP-KO mice, and co-treatment with IAP reduced inflammation to control levels. Taken together, these studies show that IAP prevents inflammation caused by UDP alone and in combination with other ligands, and the anti-inflammatory effect of IAP against UDP persists in mouse small intestines. The benefits of IAP in intestinal disease may be partly due to inhibition of the proinflammatory activity of UDP.

Keywords: lipopolysaccharide, P2Y₆ pyrimidinergic receptor, intestinal loop model
INTRODUCTION

The gut mucosal immune system is defined by complex interactions between inflammatory products of commensal and pathogenic luminal bacteria and host factors that serve both to promote a defensive inflammatory response and to protect the host from excessive inflammation (20, 38). The number of factors involved in this process is vast and relates to a broad range of diseases including infectious colitis, bacterial peritonitis, and chronic conditions such as Crohn’s disease and ulcerative colitis (1, 33). Recently, extracellular nucleotides have been implicated as proinflammatory mediators in intestinal disease (2, 14). Nucleotides play a broad range of roles in biological processes by binding to P2 receptors, of which there are two classes: P2X receptors are ligand-gated channels that respond to ATP, and P2Y receptors are G protein-coupled receptors that differ in their specificity for adenine and uridine nucleotides (44).

In inflammatory bowel disease, commensal bacteria are linked to an excessive inflammatory response, resulting in release of large amounts of nucleotides into the extracellular environment (14, 19), which has been shown to aggravate colonic inflammation in rats with chemically-induced experimental colitis (2).

The nucleotide uridine diphosphate (UDP) is the specific ligand for the P2Y<sub>6</sub> pyrimidinergic receptor, which has been directly implicated in immune function and intestinal inflammation (19, 39). P2Y<sub>6</sub> receptor mRNA is found widely, including in intestinal epithelium, lymphocytes, and macrophages (12, 22, 47), and is highly expressed in T cells infiltrating active inflammatory bowel disease (IBD) tissue but absent in the T cells of normal bowel, suggesting a role for the P2Y<sub>6</sub> receptor in the pathogenesis of IBD-mediated damage (39). In addition, P2Y<sub>6</sub> receptor mRNA is
greatly increased both in colon tissue of dextran sodium sulfate (DSS)-treated mice as well as in human biopsies of Crohn’s and ulcerative colitis tissues (19), and when gut epithelial cells are subjected to inflammatory cytokines, they release UDP into the media and upregulate expression of the P2Y\textsubscript{6} receptor (19). Activation of the P2Y\textsubscript{6} receptor has been shown to stimulate production of interleukin-8, a powerful neutrophil and monocyte chemoattractant, in a variety of cell types including human monocytes, THP-1 monocytic cells, and in cultured Caco-2/15 cells (19). The media of UDP-treated monocytes cause chemotaxis of neutrophils (24), which is prevented by treating the cells with apyrase, a class of nucleotide scavenger that is ubiquitously expressed in eukaryotes and has been found in some prokaryotes (37).

The small intestinal brush border enzyme intestinal alkaline phosphatase (IAP) is known to play a role in the interaction between the host and luminal bacteria (17, 25). IAP is expressed on the small-intestinal epithelium and is secreted bidirectionally into the bloodstream and the lumen, maintaining activity throughout the colon and into the stool (4). IAP has been shown to have a broad range of roles (17, 25) involving regulation of lipid absorption (30-32), regulation of bicarbonate secretion and duodenal luminal pH (3, 29), maintenance of normal gut microbiota (28), protection against translocation of luminal bacteria across the gut epithelium (18), and dephosphorylation and subsequent detoxification of bacterial toxins including lipopolysaccharide (LPS) (6, 18, 34), flagellin (11), and unmethylated cytosine-phosphate-guanine deoxyribonucleic acid (CpG DNA)(11). We hypothesized that IAP protects against the proinflammatory nucleotide UDP, possibly explaining the benefit that IAP confers in IBD.
MATERIALS AND METHODS

Cell culture and reagents. Human monocyte THP-1 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 culture media (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% antibiotic-antimycotic solution (Cellgro, Herndon, VA), and 0.05 mM mercaptoethanol (Sigma, St. Louis, MO). Human colorectal adenocarcinoma HT-29 cells were purchased from American Type Culture Collection and maintained in McCoy’s culture media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Lonza, Allendale, PA), 2 mM L-glutamine, and 1% antibiotic-antimycotic solution (Cellgro). Human colorectal adenocarcinoma Caco-2 cells were purchased from American Type Culture Collection and maintained in RPMI-1640 culture media (Hyclone) supplemented with 20% heat-inactivated FBS and 1% antibiotic-antimycotic solution (Cellgro). Murine macrophage RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM culture media (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% antibiotic-antimycotic solution (Cellgro). UDP (uridine 5’-diphosphate sodium salt, >96% purity by HPLC) (Sigma), LPS (E. coli serotype 055:B5) (Sigma), flagellin (ultra-pure flagellin S. Typhimurium) (Invivogen, San Diego, CA), Pam3Cys-((S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys₄-OH, trihydrochloride) (EMD Biosciences, Gibbstown, NJ), mouse TNF-α (Biolegend, San Diego, CA), potato apyrase (grade III, from S. tuberosum, Sigma), polymixin B sulfate (Sigma), TLR5 blocking antibody (rat polyclonal) (Invivogen), L-phenylalanine (Sigma), and L-homoarginine (Sigma) were reconstituted, aliquoted, and stored according to
manufacturer directions. Bovine intestinal alkaline phosphatase (Sigma) was reconstituted in the manufacturer-suggested vehicle (50 mM Tris-Hcl, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol at pH 7.9) and stored according to manufacturer directions.

*Determination of free phosphate release by malachite green assay.* The malachite green-based phosphate quantization assay was modified from Baykov et al. (7) as follows. The working solution (7 uM ammonium molybdate (Sigma), 0.2% Tween 20, 15 mM Malachite green (Sigma)) for the assay was mixed immediately before the experiment. Separately, nucleotides (10-1,000 μM) or LPS (1 mg/mL) were combined with enzymes (1-10 U/mL of IAP, potato apyrase, or Antarctic phosphatase (cloned from psychrophilic Antarctic bacterium strain TAB5, New England Biolabs, Ipswich, MA) with buffers) and enzyme inhibitors (10 μM phenylalanine or homoarginine) and incubated in 96-well plates (Costar, Corning, NY) at 37°C. At predetermined time points, working solution was added at a ratio of 1:4 (malachite green working solution: incubated groups) and optical density at 630 nm was measured after 60 minutes at room temperature and compared to a phosphate standard curve. IAP dephosphorylation of LPS was used as a positive control since this reaction has been well established by our lab and others (11, 18, 34, 41).

*In vitro inflammatory response to UDP and other proinflammatory ligands.* THP-1 cells were plated at a concentration of 1 x 10⁶ cells/mL. HT-29 cells were seeded into 48-well plates and grown until 80% confluence. Caco-2 cells were seeded into 48-well plates and grown until 4 days post-confluency. UDP (0 to 1,000 μM final concentration), LPS (100 ng/mL), flagellin (100 ng/mL), TNF-α (100 ng/mL), and Pam3Cys (100 ng/mL)
were incubated at 37°C for 1 hour +/- IAP (0-400 U/mL) or potato apyrase (1 U/mL) or polymixin B (10 μg/mL). Incubation solutions were then applied directly to cells. In some experiments a blocking TLR5 antibody (5 μg/mL) was applied to cells 15 minutes prior to application of inflammatory ligands. After six hours of incubation, media were collected and measured for IL-8 by ELISA (BD Biosciences, San Diego, CA). Of note, UDP dosing was based on previous studies using UDP in cell culture (13, 19, 46) as well as the finding by Grbic et al. that Caco-2 cells exposed to inflammatory-like stress release approximately 100 nM of UDP (19).

**Animals.** IAP-KO mice (Akp3-/-, *Mus musculus* C57BL/6)(32) were obtained from the Sanford-Burnham Medical Research Institute (La Jolla, CA) and bred at the Massachusetts General Hospital (MGH, Boston, MA) animal facility to create homozygous IAP-KO, heterozygous, and wild-type C57BL/6 (WT) littermates. Genotype was confirmed by polymerase chain reaction analysis (32). Animals were maintained in a specific pathogen-free environment at MGH in accordance with the guidelines of the Committee on Animals of Harvard Medical School (Boston, MA). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and according to regulations of the Subcommittee on Research Animal Care of the MGH and the National Institutes of Health (publication no. 85-23, NIH, 1985).

**Intestinal Loop Model.** We established a novel surgical intestinal loop model to study the impact of IAP on the inflammatory activity of UDP within the small intestines. Mice were anesthetized with 3.0% inhaled isoflurane (Baxter International, Deerfield, IL) and intraperitoneal pentobarbital (40 mg/kg, Akorn Inc., Lake Forest, IL) and were kept
on a heating pad at 37ºC. After midline laparotomy, the proximal jejunum was identified and a 5cm loop was isolated by ligating either end with 5-0 silk suture (Ethicon Inc., USA). 100 µl of either endotoxin-free water or UDP (1 mM), with or without IAP (1000 U/mL), was instilled into the loop using a 28 gauge needle. The incision was closed in two layers using 3-0 silk suture (Ethicon Inc.). Two hours later, the incision was reopened and the loop was harvested. The luminal contents were isolated, and the intestinal tissue was snap frozen in liquid nitrogen and stored at -80ºC for later analysis.

In vitro cytokine response to luminal contents and UDP. To study the impact of endogenous IAP on the inflammatory activity of UDP within the in vivo environment, UDP (1 mM) or endotoxin-free water was instilled into the loop model of IAP-KO mice and WT littermates as described above (5 mice per group). The luminal contents were then collected, proportionally diluted in endotoxin-free water, and centrifuged at 13,000g for 15 minutes twice, and the supernatant was collected. RAW264.7 cells were plated at a concentration of 2x10⁶ cells/ml and starved for 8 hours in DMEM with 0.5% FBS. Cells were then treated with 100 µl of luminal content supernatant. After overnight incubation, media were collected and assayed for TNF-α concentration by ELISA (eBioscience, San Diego, CA).

In vivo cytokine response to luminal UDP. To study the action of IAP against UDP-induced inflammation in the small intestines, jejunal loops were created in IAP-KO mice as described above, and either UDP (1 mM) or endotoxin-free water was instilled ± IAP (100 U) (11 mice in each UDP group, 5 mice in each water group). The intestinal loops were harvested after two hours and homogenized with 10 volumes of ice-cold RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% NP-
40, 10 mM EDTA, 0.1% SDS, including both protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) followed by 30 minutes of incubation on ice. The homogenates were centrifuged (15000 g for 15 minutes at 4°C, twice), and the supernatants were collected. Protein concentration was determined using the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific), and TNF-α was quantified by ELISA (eBioscience).

Statistical analyses. Malachite green assay experiments were carried out in duplicate or triplicate sample wells, expressed as averages with standard error, and repeated three times. Cell culture results were averaged between duplicate or triplicate wells, expressed with standard error, and repeated a minimum of three times. Data presented are of representative experiments. Animal experiments were performed on a minimum of 5 mice per group. Data are expressed as mean +/- SE. Statistical significance was determined by unpaired student’s t-test using Excel software (Microsoft® Office 2007).
**RESULTS**

*Dephosphorylation of UDP by IAP is efficient, dose-dependent, and specific.* We first examined the ability of IAP to dephosphorylate UDP using the malachite green assay for free phosphate measurement. It has previously been shown by other methods that alkaline phosphatases release inorganic phosphate from a variety of organic pyrophosphate substrates (16). Dephosphorylation of UDP and LPS by IAP was found to occur in a dose-dependent fashion with a 63-fold increase in free phosphate released from UDP by IAP (10 U/mL) after 60 minutes (Fig. 1A, p < 0.05) and a 2.9-fold increase from LPS (1 mg/mL) by IAP (10 U/mL) (Fig. 1B, p < 0.05). As each mole of UDP contains two moles of phosphate, release of greater than 100 uM of free phosphate was not a surprise. In addition, treatment with L-phenylalanine (10 mM), a specific inhibitor of IAP (18), prevented IAP (10 U/mL) from dephosphorylating UDP (Fig. 1C, 74% decrease in free phosphate compared to IAP treatment, p < 0.05) and LPS (Fig 1D, 55% decrease compared to IAP treatment, p < 0.005). Homoarginine, an inhibitor of non-intestinal alkaline phosphatases, did not affect dephosphorylation of either UDP (Fig. 1C, p > 0.3) or LPS (Fig. 1D, p > 0.1) by IAP, confirming that the dephosphorylation was specific to IAP rather than contaminants. Other phosphatases were used as positive controls and were effective but less efficient at dephosphorylating UDP. Potato apyrase, which hydrolyzes extracellular nucleoside triphosphates and diphosphates, caused a 20-fold increase in phosphate release from UDP, and Antarctic alkaline phosphatase, a bacterial-derived alkaline phosphatase, caused a 15-fold increased in phosphate release from UDP (p < 0.05 for both). These enzymes were
unable to dephosphorylate LPS (p > 0.4 for both), demonstrating a specificity of IAP for LPS.

IAP prevents the proinflammatory effect of UDP on immune cells and intestinal cells. First, we confirmed that UDP causes dose-dependent activation of the inflammatory pathway in undifferentiated THP-1 monocytes, which was shown by Warny et al. (46) but not reproduced by others (13). Increasing concentrations of UDP resulted in increased release of IL-8, an effect that peaked at a concentration of 100 μM UDP (Fig. 2A, p < 0.001). Above 1,000 μM we saw significant cell swelling and cell death, indicating toxicity of the nucleotide. Next, we showed that on an immune cell line and on two separate colon cancer cell lines, the proinflammatory effect of UDP was prevented by pretreatment with IAP, again in a dose-dependent fashion. On undifferentiated THP-1 cells (Fig. 2B), treatment with UDP resulted in a 4.7-fold increase in IL-8 release compared to control, and co-treatment with IAP (20 U/mL) resulted in a >50% decrease in IL-8 release (p < 0.001). On HT-29 cells (Fig. 2C), treatment with UDP resulted in a 2.2-fold increase in IL-8 release, and co-treatment with IAP (20 U/mL) decreased IL-8 release by >35% (p < 0.001). On Caco-2 cells (Fig. 2D), UDP treatment caused a 3.4-fold increase in IL-8 release, which was completely inhibited by co-treatment with IAP (20 U/mL) (p < 0.001). Potato apyrase, a nucleotide scavenger, was used with each cell line as a positive control and also resulted in statistically significant UDP inhibition (p < 0.01 for all cases).

IL-8 release due to inflammatory ligands is enhanced by the presence of UDP, and this effect is prevented by IAP. Others have shown that blocking the P2Y$_6$ receptor decreases IL-8 release induced by LPS (24, 46) and Pam3Cys (8). Here we showed
that addition of UDP to THP-1 cell cultures exposed to proinflammatory ligands resulted in a significant amplification of inflammation as measured by IL-8 release, including for LPS (Fig. 3A, 1.7-fold increase over LPS alone, p < 0.001), flagellin (Fig. 3B, 1.7-fold increase over flagellin alone, p < 0.001), Pam3Cys (Fig. 3C, 1.4-fold increase over Pam3Cys alone, p < 0.001) and TNF-α (Fig. 3D, 3.9-fold increase over TNF-α alone, p < 0.001). This enhanced IL-8 release was prevented most notably by IAP (70% decrease for LPS + UDP, 60% decrease for flagellin + UDP, 42% decrease for Pam3Cys + UDP, 79% decrease for TNF-α + UDP, p < 0.001 for all ligands) but also by known inhibitors of these inflammatory ligands (91% decrease due to polymixin B for LPS + UDP, p < 0.001; and 41% decrease due to TLR5-blocking antibody for flagellin + UDP, p < 0.001) as well as potato apyrase (72% decrease for LPS + UDP, 22% decrease for flagellin + UDP, 40% decrease for Pam3Cys + UDP, and 66% decrease for TNF-α + UDP, p < 0.001 for all ligands). As we have previously shown (11), IAP was ineffective at blocking the effects of either TNF-α or Pam3Cys alone. However, when used in combination with UDP, IAP did exert substantial inhibitory effects.

\textit{IAP is highly efficient at preventing inflammation caused by UDP alone as well as the synergistic effect of LPS and UDP.} In attempts to determine the principal target of IAP in preventing the enhanced inflammation caused by the combination of UDP and other inflammatory ligands, we analyzed the relative effect of increasing doses of IAP on IL-8 release by THP-1 cells treated with LPS and UDP (Fig. 4). We found that relative to the conditions of cells treated with vehicle only, IAP was the most effective at preventing inflammation caused by UDP alone with a statistically significant decrease in IL-8 release achieved with 10 U/mL of IAP (p < 0.001) and with 500 U/mL resulting in an
approximately 70% decrease in IL-8 release (p < 0.001). The pattern of decreased IL-8 release in the cells treated with LPS + UDP was similar to that of UDP alone, with 500 U/mL of IAP resulting in an approximately 60% decrease in IL-8 release (p < 0.001), whereas LPS alone was relatively resistant to IAP treatment, with 500 U/mL of IAP causing a 13% decrease in IL-8 release (p < 0.01).

Endogenous mouse intestinal IAP prevents UDP-induced inflammation. To establish that endogenous IAP in the mouse small intestine could inhibit the proinflammatory action of UDP, we incubated UDP in a small intestinal loop model in both WT and IAP-KO mice, collected the luminal contents, and applied them to mouse RAW264.7 macrophage cell culture. We found that luminal contents from WT mice treated with UDP induced a greater than 3-fold increase in TNF-α compared to luminal contents treated with water alone (p < 0.001) (Fig. 5). Interestingly, IAP-KO luminal contents treated with UDP caused 25% more TNF-α release than WT luminal contents treated with UDP (p=0.01). There was no significant difference in TNF-α release between IAP-KO and WT contents treated with endotoxin-free water (p=0.19).

IAP prevents mouse small intestinal inflammation caused by luminal UDP. To assess the direct proinflammatory properties of UDP on small intestinal tissue, we incubated UDP in the small intestinal loop model using IAP-KO mice, harvested the intestinal loops, washed off the luminal contents, homogenized the tissue, and measured mouse TNF-α by ELISA. We found that treatment with intraluminal UDP caused a greater than 2-fold increase in intestinal TNF-α (p=0.02) and that co-treatment with UDP and IAP caused a 37% decrease in TNF-α release compared to UDP alone (p=0.03), resulting in similar TNF-α release as with water alone (p=0.08) and IAP alone.
Treatment with IAP alone caused no significant change in TNF-α 
(p=0.28) compared to treatment with water.
It is well established that IBD in part results from an inappropriate activation of the mucosal immune system driven by the presence of luminal bacteria (20, 33). As evidence, several animal models that normally develop spontaneous colitis due to genetic alterations in their immune systems remain colitis-free under germ-free conditions (15, 38). Similarly, in human patients with IBD, clinical improvement is seen with diversion of the fecal stream (21) and broad-spectrum antibiotic treatment (40).

The host response to bacterial ligands such as LPS and flagellin is complex and has been characterized in a variety of \textit{in vitro} and \textit{in vivo} model systems (36, 43, 45). While it is clear that bacteria are critical to induction of gut inflammation in experimental models of IBD, various bacterial ligands are generally studied in isolation, whereas in reality they interact with each other and with multiple host factors.

We have been interested in exploring the effects of the gut enzyme IAP in the context of intestinal inflammation. Work in our lab and others has established a protective role of IAP in animal models of local intestinal inflammation as well as systemic sepsis secondary to abdominal processes. In acute (9, 42) and chronic (35) rodent models of colitis, exogenous administration of IAP significantly reduced colonic inflammation, and Campbell et al. both induced and inhibited endogenous IAP activity in mice with DSS-colitis and showed that disease severity negatively correlated with IAP activity (10). Additionally, in the first report of IAP for human IBD, Lukas et al. showed that intraduodenal administration of IAP in 21 patients with refractory ulcerative colitis resulted in significant improvement in several objective disease parameters. Importantly, IAP was associated with no significant side effects (27), unlike nearly every
other treatment available for IBD (33). Taken together, these studies indicate a protective role for both endogenous and exogenous IAP against colitis.

We and others have shown that IAP is able to dephosphorylate and detoxify several bacterial toxins, but its precise mechanism of action in disease models remains poorly understood. The known bacterial ligands that are targets of IAP cause inflammation through binding to specific Toll-like receptors, which are located on the cell-surface membrane (1). In this study we considered that the process may be more complex than simple dephosphorylation of these toxins. With UDP as a known mediator of cytokine release from immune cells and gastrointestinal epithelial cells (19) and also playing a dramatic role in upregulating neutrophil migration in response to bacterial inflammatory ligands (8, 24), it is conceivable that a cycle exists in which inflammation is initiated by bacterial ligands, leading to release of extracellular nucleotides such as UDP as danger signals, thus further amplifying inflammation. Interestingly, Lavoie et al. (26) demonstrated localization of a family of membrane-bound ectonucleotidases, the nucleoside triphosphate diphosphohydrolases (NTPDases), to the epithelium of the foregut as well as the entire enteric nervous system. Kukulski et al. (23) showed that one member of this family of ectonucleotidases, NTPDase1, controls IL-8 production by human neutrophils through regulation of P2Y receptor activation, demonstrating a clear relationship between dephosphorylation of luminal nucleotides and intestinal inflammation. The NTPDase family of enzymes has a similar function of hydrolysis of nucleotides as we have demonstrated in the case of IAP, a secreted enzyme that maintains activity throughout the entire small intestine and colon.
Exogenous IAP has been shown to be a potential treatment in both human ulcerative colitis (27) and in several animal models of IBD. Though there are clear anti-inflammatory properties of IAP in a variety of bacterial-induced gastrointestinal diseases, its precise mechanism of action is unclear. Here we show that IAP efficiently dephosphorylates and detoxifies the nucleotide UDP, effectively preventing the primary inflammation caused by UDP on immune and epithelial cells and also preventing the synergistic inflammation that UDP causes in the presence of other inflammatory ligands. This effect persisted in vivo in a jejunal loop model, with WT mouse luminal contents more effective at detoxifying UDP than IAP-KO luminal contents, and with IAP-KO intestinal tissues treated with UDP releasing lower levels of TNF-α when luminal IAP activity was restored. This demonstrates both that the proinflammatory effects of UDP carry over into animal models, and that IAP is effective at preventing UDP-induced inflammation in vivo, though further studies with inhibitors of IAP, UDP, and the P2Y$_6$ receptor would strengthen this conclusion. While it is well established that IAP detoxifies LPS and other bacterial ligands, high doses of IAP were required to detoxify bacterial ligands. On the contrary, IAP efficiently prevents inflammation due to UDP. This suggests that the anti-inflammatory action of IAP in the complex environment of the intestines could be related to its ability to remove inflammatory signals such as extracellular nucleotides that are derived from the host rather than from bacteria themselves. Interestingly, IAP prevents the synergistic inflammation that results from the combination of UDP with ligands that are not directly affected by IAP, such as TNF-α and Pam3Cys. It is known that in response to inflammatory stimuli, host cells release nucleotides including UDP (19), which cause both release of inflammatory cytokines
and promote neutrophil migration. Despite the finding that the decreased inflammation provided by IAP against UDP alone is small in absolute terms and that inflammation caused by LPS could only be prevented with high doses of IAP, the anti-inflammatory effect of IAP against the combination of LPS and UDP was more profound than the action of IAP against either ligand alone. This supports the conclusions by others that host-derived nucleotides act in synergy with bacterial ligands to regulate inflammation (5, 46) and is the first demonstration of IAP working against a host-derived inflammatory factor.
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REFERENCES


**FIGURE LEGEND**

**Fig. 1.** IAP specifically and efficiently dephosphorylates UDP.  (A & B) UDP (100 μM) or LPS (1 mg/mL) was combined with increasing doses of IAP or vehicle and incubated for 1 hr at 37°C.  Malachite green working solution was added at a ratio of 1:4 (malachite green working solution : incubation volume) and OD 630 nm was read after 60 minutes.  Incubation with IAP resulted in a dose-dependent increase in free phosphate for both UDP (63-fold increase from UDP by 10 U/mL IAP) and LPS (2.9-fold increase from LPS by 10 U/mL IAP).  *, p < 0.05; ***, p < 0.001 as compared with no IAP.  (C & D) UDP (100 μM) or LPS (1 mg/mL) was combined with apyrase (5 U/mL), Antarctic phosphatase (5 U/mL), or IAP (5 U/mL) +/- phenylalanine (10 mM) or homoarginine (10 mM).  After 1 hr incubation, free phosphate concentration was determined by malachite green reaction.  Phenylalanine prevented release of free phosphate by IAP for both UDP (approx. 75% decrease in free phosphate compared to IAP treatment, p < 0.05) and LPS (approx. 55% decrease compared to IAP treatment, p < 0.005), and neither were significantly affected by homoarginine.  Apyrase and Antarctic phosphatase released free phosphate from UDP (20-fold and 15-fold increase, respectively, p < 0.05 for both) but not significantly from LPS.  Results shown are representative of three experiments.

**Fig. 2.** The proinflammatory effects of UDP are prevented by exogenous IAP on three separate cell lines.  (A) Increasing doses of UDP were applied to THP-1 monocytic cells for 6 hr, and media were assayed for IL-8 by ELISA.  UDP increased release of IL-8 in a
dose-dependent fashion, which peaked at 100 μM, above which cell toxicity was seen.

(B-D) Treatment with UDP resulted in increased IL-8 release from THP-1 cells (approx. 5-fold), HT-29 cells (>2-fold), and Caco-2 cells (approx. 3.5-fold; p < 0.001 for all). Co-treatment with IAP (20 U/mL) decreased IL-8 release by >50% (B), >35% (C), and >70% (D), respectively (p < 0.001 for all). Apyrase (1 U/mL) similarly prevented UDP-induced IL-8 release (p < 0.01 for all cell lines). Results shown are representative of three or four experiments. ^^^, p < 0.001 as compared with control, and *, p < 0.05; ***, p < 0.001 as compared with UDP stimulation.

Fig. 3. UDP enhances in vitro release of IL-8 due to other proinflammatory ligands, and this effect is prevented by exogenous IAP. UDP (100 μM) or other proinflammatory ligands (100 ng/mL) (A: LPS, B: flagellin, C: Pam3Cys, D: TNF-α) were preincubated with IAP (200 U/mL) or apyrase (1 U/mL) and then applied directly to THP-1 monocytes. Specific inhibitors of LPS (polymixin 10 μg/mL) and the flagellin receptor (TLR5 antibody, 5 μg/mL) were also used. After 6 hr of incubation, media were assayed for IL-8 by ELISA. Presence of UDP significantly enhanced IL-8 release due to other inflammatory ligands (p < 0.001), most notably with LPS and TNF-α. Exogenous IAP and apyrase prevented this increase in IL-8 release (p < 0.001) even in cases where IAP had no effect on IL-8 release caused by the primary inflammatory ligand (C & D: Pam3Cys and TNF-α). Results shown are representative of four experiments. ^, p < 0.001 as compared with UDP alone and LPS, flagellin, Pam3Cys, or TNF-α alone respectively, and *, p < 0.001 as compared with vehicle group.
Fig. 4. Prevention of enhanced IL-8 release due to combination of LPS and IAP is largely due to the effect of exogenous IAP on UDP. UDP (100 μM) and LPS (100 ng/mL) or the combination of the two were treated with increasing doses of IAP (0-500 U/mL) for 1 hr then applied to THP-1 monocytes. Media were assayed for IL-8 by ELISA after 6 hr. Results are normalized to the vehicle treatment for each group. While IL-8 release due to LPS was relatively resistant to exogenous IAP, IL-8 release due to UDP treatment and UDP+LPS treatment was efficiently prevented by exogenous IAP treatment, suggesting that the anti-inflammatory action of IAP was weighted towards UDP dephosphorylation. Results shown are representative of three experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 as compared with vehicle group.

Fig 5. Luminal UDP is proinflammatory on target cells, more so in IAP-KO mouse luminal contents than in WT mouse luminal contents. 100 μl of UDP (1 mM) or water was instilled into a 5-cm jejunal loop in IAP-KO or WT mice (n=5 mice per group). After 2 hr, the luminal contents were collected and applied to RAW264.7 cells, and after 16 hr the supernatant was assayed for TNF-α by ELISA. UDP-treated luminal contents of both WT and IAP-KO mice caused more TNF-α release by target cells than control luminal contents (>3-fold increase for WT mice, p < 0.001). IAP-KO luminal contents treated with UDP caused 25% more TNF-α release than WT luminal contents (p=0.01). TNF-α release due to IAP-KO and WT contents treated with endotoxin-free water were similar (p=0.19). **, p < 0.01, and ***, p < 0.001.
Fig 6. Exogenous IAP prevents intestinal inflammation caused by luminal UDP in IAP-KO mice. 100 ul of UDP (1 mM) or water, +/- IAP (1000 U/mL) was instilled into a 5cm jejunal loop in IAP-KO mice (n=11 mice per UDP group, n=5 mice per water group). After 2 hr, the intestinal loops were collected and homogenized, and TNF-α content was measured by ELISA. UDP treatment caused a >2-fold increase in intestinal TNF-α (p=0.02), and co-treatment with UDP and IAP prevented TNF-α release, resulting in similar levels as with water (p=0.08) and IAP (p=0.46) alone. Treatment with IAP alone was similar to treatment with water (p=0.28). *, p < 0.05.
**Fig. 1 A**

UDP 100μM

**Fig. 1 B**

LPS 1mg/mL

**Fig. 1 C**

UDP 100 μM

**Fig. 1 D**

LPS 1mg/mL

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Fig. 4

Relative IL-8 release

IAP (U/mL)

UDP  LPS  LPS + UDP

*  **  ***  ***  ***  ***
Fig. 5

The figure shows a bar graph comparing TNF-a (pg/mL) in cell media between WT and IAP-KO conditions after treatment with Water or UDP. The graph indicates significant differences between the groups, with IAP-KO showing a higher TNF-a level compared to WT, especially in the UDP treatment condition.
Fig. 6

TNF-α in intestinal tissue (pg/mg protein)

- Water
- IAP
- UDP
- UDP + IAP

* * *