

Anti-inflammatory effects of inosine in human monocytes, neutrophils and epithelial cells *in vitro*

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Abstract. Inosine is an endogenous purine, which has been recently shown to exert immunomodulatory, anti-inflammatory and anti-shock effects in rodent experimental systems. Some of these actions may be related to partial adenosine receptor agonistic effects. It has not been investigated previously whether inosine exerts similar immunomodulatory or anti-inflammatory effects in human cells or enzymes. Here we investigated the effects of inosine on the activation of human monocytes, neutrophils and epithelial cells *in vitro*. Furthermore, using a human inosine-5'-monophosphate dehydrogenase (IMPDH) enzyme, we examined the potential effects of inosine on the activity of IMPDH, an enzyme involved in the regulation of certain inflammatory/immune processes. Tumor necrosis factor α (TNF- α) production of bacterial lipopolysaccharide (LPS) stimulated whole blood was used as an indicator of human monocyte activation. The response was dose-dependently, partially suppressed in the presence of inosine. Inosine exerted a dose-dependent and, at the highest dose (3 mM), complete inhibition of the ability of human neutrophils activated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) to induce cytochrome C reduction *in vitro*. In the human colon cancer cell line HT-29, inosine dose-dependently attenuated the production of IL-8. Inosine failed to affect the activity of IMPDH. Taken together, we conclude that inosine exerts anti-inflammatory effects in many human cell types. Further studies need to establish whether inosine supplementation exerts anti-inflammatory effects in human beings.

Introduction

Inosine is an endogenous purine nucleoside, which is formed during the breakdown of adenosine by adenosine deaminase

(1,2). This molecule is released into the extracellular space from cells upon metabolic stress or from the sympathetic nervous system (1,2). In ischemic tissues, inosine concentration can increase to levels as high as 1-3 mM and increased tissue inosine levels are present in various inflammatory states (1,2). Until recently, a widely held belief was that inosine is an inert metabolite of adenosine, which does not affect the underlying pathophysiological processes. However, in a recent study in a murine experimental system we showed that inosine inhibits pro-inflammatory cytokine and chemokine production *in vitro* and *in vivo*, and enhances the production of the anti-inflammatory IL-10 via a post-transcriptional mechanism (3). Subsequent *in vivo* studies in rodents demonstrated reduced endotoxin-induced mortality in mice (3) and improved organ function and vascular patency in rodent models of endotoxic shock and polymicrobial sepsis (3-5). In addition, a recent study demonstrated reduced tumor necrosis factor α (TNF- α) and macrophage inflammatory protein 2 (MIP-2) production in the presence of inosine in reperfused skeletal muscle, coupled with protection against the degree of reperfusion injury (6).

As all of the above listed studies utilized murine experimental systems, the question remained as to whether inosine exerts immunomodulatory/anti-inflammatory effects in human cells and enzyme systems. This issue is especially relevant, as the potential utility of inosine to treat human disease has been suggested recently (4-9).

Materials and methods

Human whole blood collection and isolation of human neutrophils. Human whole blood was collected aseptically from healthy volunteers into sterile tubes containing heparin (10 units/ml blood). The volunteers gave written consent to participate in this study. The monocytes, which produce TNF- α in human whole blood, were stimulated using *E. coli* LPS (10 ng/ml) for 4 h, followed by measurement of TNF- α concentrations in the plasma as previously described (10). Human neutrophils were isolated by dextran-Percoll separation from whole blood. Six ml of 6% dextran solution was added to 20 ml samples of blood, and the blood/dextran mixture was allowed to stand for 30 min at room temperature. The supernatant was mixed with cold Hanks balanced salt solution and allowed to stand an additional 20 min on ice, followed by centrifugation (200 x g, 10 min, 4°C). The pellet was

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re-suspended into 1 ml Hanks balanced salt solution, and the cell suspension was layered over a Percoll gradient (1.5 ml of each density; 1.04, 1.08, 1.10) and then centrifuged (600 x g, 15 min, room temperature). The interface of cells between 1.04 and 1.08 layers was collected and washed by centrifugation (200 x g, 10 min, 4°C) three times with Hanks balanced salt solution, then the cells were used for superoxide anion determination experiments.

Measurement of TNF- α production in endotoxin-stimulated human whole blood. Whole blood experiments were performed by adding 50 μ l of LPS directly to 48-well culture plates containing 400 μ l heparinized whole blood. Following incubations in the presence of various concentrations of inosine (0.3-3 mM) for 3 h, the culture plates were centrifuged (900 x g, 10 min, 4°C) and the plasma or the culture supernatants were removed and stored at -80°C. TNF- α levels were quantitated using a human TNF- α ELISA kit (R&D Systems, Minneapolis, MN, USA).

Superoxide anion assay in fMLP-stimulated human neutrophils. Superoxide anion production was determined by a modification of a method utilizing superoxide dismutase-inhibitable reduction of cytochrome C (11). Neutrophils (4x10⁶ cells) suspended in Hanks balanced salt solution were incubated with inosine (Sigma Chem. Co., St. Louis, MO, USA) or vehicle for 1 h in a total volume of 0.2 ml. Then 0.8 ml of Hanks balanced salt solution containing Ca²⁺ and Mg²⁺ supplemented with 1 μ M fMLP (Sigma Chem. Co., St. Louis, MO, USA) and 50 μ M cytochrome C (horse heart type VI; Sigma Chem. Co., St. Louis, MO, USA) was added to the cell suspension and incubated in the presence or absence of inosine (0.3-3 mM) for an additional 10 min. After centrifugation (2000 x g, 4°C), the absorbance of the supernatant was read at 550 nm with a spectrophotometer. The change in absorbance was converted to nanomoles of cytochrome C reduced and expressed as nanomoles O₂/10⁶ neutrophils.

Measurement of IL-8 production in the HT-29 human epithelial cell line. One of the major mediators of immunostimulated epithelial cells is IL-8, which is involved in the recruitment of mononuclear cells into the site of inflammation. HT-29 cells (ATCC; Rockville, MD) between passages 5 and 15 were cultured in modified McCoy's 5A medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY). Cells in the presence or absence of various concentrations of inosine (0.3-3 mM) were stimulated with human IL-1 β (20 ng/ml) to induce the production of IL-8. IL-8 levels were quantitated using a human IL-8 ELISA kit (R&D Systems, Minneapolis, MN, USA).

Human IMPDH activity measurements in vitro. Expression plasmid pET 23a (Novagen) containing human type II IMPDH protein coding sequences (12) was a kind gift of Dr F. Collart at Argonne National Laboratories (Argonne National Laboratory, Argonne, IL 60439, USA). IMPDH was expressed in *E. coli* and purified as follows: bacterial strain (*E. coli* BL21) carrying these recombinant plasmids were grown at 37°C in Luria broth containing ampicillin (100 μ g/ml) to an A600 of 0.5

and then induced for 3 h with 0.5 mM IPTG. Following induction, bacteria were harvested and washed with STE buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA). Cells were suspended in buffer A (20 mM Tris, pH 8.0, 0.1 mM EDTA and 1 mM PMSF) containing leupeptin (5 μ g/ml) aprotinin (10 μ g/ml) and 150 mM KCl. After two freeze thaws, the cells were sonicated in an ice bath until lysis was complete and clarified by centrifugation at 15,000 g for 30 min. The soluble lysate was loaded onto a Blue-Sepharose column (Pharmacia) equilibrated with buffer A containing 150 mM NaCl. After washing the column with same buffer IMPDH was eluted with a 0.15-1.0 M NaCl gradient in buffer A. Active fractions were pooled, dialyzed against buffer A containing 50 mM KCl and then loaded onto a Q-Sepharose column. IMPDH enzyme was eluted from the column by using a linear gradient of 0.05-1.0 M KCl in buffer A. Fractions with high IMPDH activity were pooled and dialyzed against PBS and stored at -70°C. The purity of eluted protein (5 μ g) was analyzed by SDS-polyacrylamide gel electrophoresis and protein concentrations were estimated by the Bradford method.

IMPDH activity was measured at 37°C using a spectrophotometric assay based on the reduction of NAD⁺ (13). IMPDH activity was determined from the rate of NADH production by monitoring the increase in absorbance at 340 nm. Readings were taken every 30 sec for 15 min. A SpectraMAX 250 spectrophotometer with a computerized kinetic measurement program was used. The assay buffer consisted of 0.1 M Tris-Cl (pH 8.1), 0.1 M KCl, 3 mM EDTA, 0.2 mM NAD⁺ and 0.2 mM IMP. The reactions were initiated by adding 25 μ l IMPDH from the stock solution (0.4 μ g protein/ml). Assays were run in triplicate the presence of inosine (0.4-5 mM), the positive control compound mycophenolic acid (MPA, 0.04-1 μ M), and appropriate vehicle controls.

Data analysis. Data are shown as mean \pm SEM. For statistical analyses of *in vitro* data one-way ANOVA was followed by Tukey's post-hoc tests. P<0.05 was considered statistically significant.

Results

Inosine attenuates endotoxin-induced TNF- α production in human whole blood in vitro. In human whole blood assay, inosine dose-dependently and partially protected against endotoxin-induced TNF- α production (Fig. 1).

Inosine blocks fMLP induced cytochrome C oxidation in human neutrophils in vitro. In human isolated neutrophils, inosine dose-dependently and, at 3 mM, completely suppressed the fMLP-induced cytochrome C response (Fig. 2).

Inosine does not affect IL-1 β induced IL-8 production in human epithelial cells in vitro. In the human intestinal epithelial cell line, inosine dose-dependently suppressed IL-1 β induced IL-8 production, with approximately 50% inhibition at 3 mM (Fig. 3).

Inosine does not affect IMPDH activity in vitro. Inosine failed to affect the activity of IMPDH *in vitro*, whereas the positive

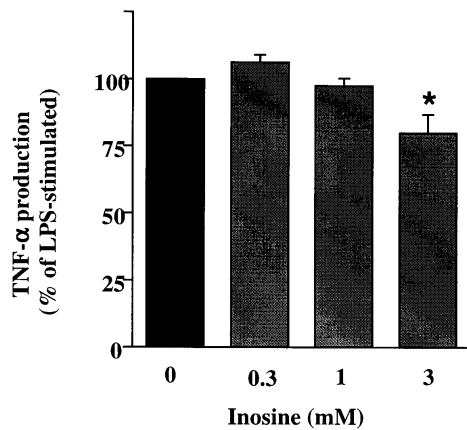


Figure 1. Inosine attenuates TNF- α production in LPS-stimulated human whole blood (n=6-8 determinations for each condition). LPS stimulated TNF- α production amounted to 276 \pm 47 pg/ml and was considered 100%. *P<0.05 indicates significant inhibition of TNF- α production by inosine.

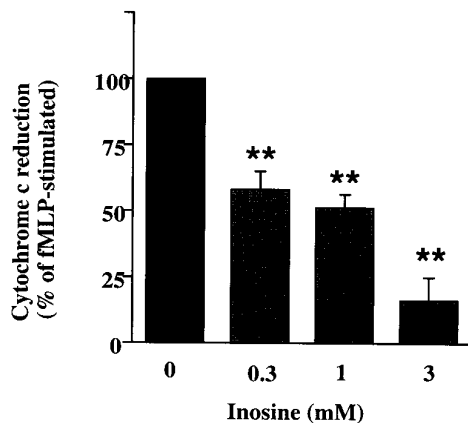


Figure 2. Inosine blocks superoxide-mediated cytochrome C oxidation in fMLP-stimulated human neutrophils (n=6-8 determinations for each condition). *P<0.05 and **P<0.01 indicate significant inhibition of cytochrome oxidation by inosine.

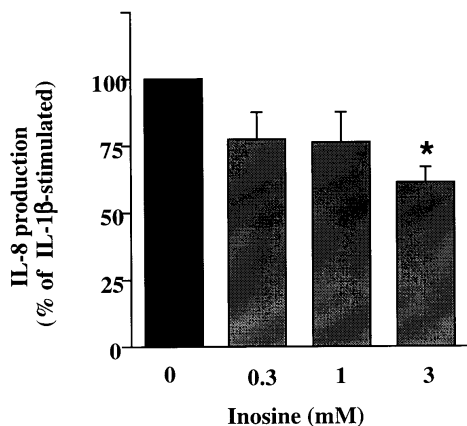


Figure 3. Inosine attenuates IL-8 production in IL-1 β -stimulated human epithelial cell line (n=6 determinations for each condition). IL-1 β stimulated IL-8 production amounted to 17 \pm 3 ng/ml and was considered 100%. *P<0.05 indicates significant inhibition of IL-8 production by inosine.

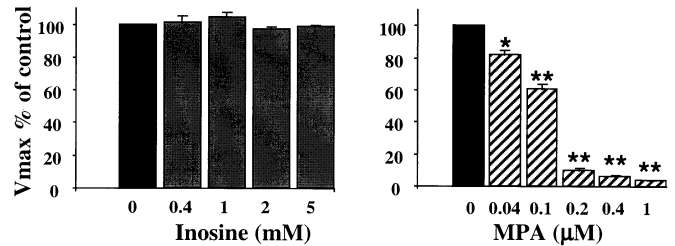


Figure 4. Lack of effect of inosine on human IMPDH activity *in vitro*. *P<0.05 and **P<0.01 indicate significant inhibition of IMPDH activity by the positive control immunosuppressive agent MPA.

compound MPA exerted a dose-dependent, potent inhibitory effect (Fig. 4).

Discussion

The studies in human cells conducted herein confirm and extend previous studies on the anti-inflammatory effect of the compound in mononuclear and epithelial cells. It is noteworthy, however, that the extent of inhibition of TNF- α production in human monocytes observed in the current study was less pronounced than the effects previously reported in murine peritoneal macrophages (3). This difference may be related to a difference between monocyte vs. macrophage responsiveness, or possibly a species-related effect. It is noteworthy that some of the cellular actions of inosine are probably related to A2a and A3 receptor partial agonism (14-16), and there are substantial differences between rodent and human adenosine receptors, which yield considerable differences in ligand-selectivity. We did not observe a cell-type difference in the effect of inosine: inosine also suppressed IL-8 production in the human epithelial cell line, HT-29. It must be pointed out that the latter cell line is a transformed human colon cancer cell line, which, in many other respects, responds markedly differently to immunological and inflammatory stimuli than freshly isolated normal human cells.

Recent study has demonstrated that inosine suppresses neutrophil recruitment into inflamed organs *in vivo* (4,5,7). Also, a derivative of inosine, inosine monophosphate (IMP), and, to a lesser extent, inosine itself, have been found to inhibit cytokine-initiated neutrophil infiltration *in vivo* and neutrophil rolling, as measured in microvessel preparations in rodents (17). Using labeled IMP it was demonstrated that IMP specifically binds to neutrophils. IMP also stimulated binding of γ -[(35)S]thio-GTP, further suggesting that IMP is a potent regulator of neutrophils (17). The current findings, demonstrating the suppression of neutrophil-mediated cytochrome reduction by inosine, extend these observations and suggest that inosine is able to attenuate superoxide production from activated neutrophils. Whether this effect may contribute to the inhibitory effect of inosine on neutrophil recruitment *in vivo* is not known. Nevertheless, it is noteworthy in this context that superoxide and its reaction product peroxynitrite, when endogenously produced in neutrophils, are able to up-regulate neutrophil adhesion receptors and thus may enhance neutrophil-endothelial interactions (18,19).

Neutrophil over-activation, and the release of neutrophil-derived free radicals and oxidants is a well known cause of multiple organ failure in circulatory shock and inflammatory bowel disease (20-22). The current findings, demonstrating the suppression of neutrophil-mediated oxidant burst by inosine may provide an additional mode of inosine's recently observed (3-6) protective action against organ failure in shock and inflammation. Also, the suppression of IL-8 by inosine in intestinal epithelial cells, coupled with previous data demonstrating that inosine suppresses chemokine production *in vitro* and *in vivo* (3-6) raises the possibility that inosine may suppress epithelial over-activation and neutrophil and macrophage recruitment into the inflamed gut. Thereby, inosine may affect the course of colitis or inflammatory bowel disease. In fact, preliminary data demonstrate that oral treatment with inosine suppresses the severity of disease in a murine model of dextrane sulfate solution induced colitis (unpublished data). The current finding that inosine exerts potent effects not only in rodent systems, but also in human neutrophils, macrophages and epithelial cells may indicate that inosine may have the potential of down-regulating neutrophil responses in human conditions associated with neutrophil, macrophage and/or epithelial inflammatory over-activation.

The enzyme IMPDH is a homotetramer of approximately 55 kDa subunits and consists of a (β/α) (8) barrel core domain and a smaller subdomain. The active site has binding pockets for the two substrates IMP and NAD. The enzymatic reaction of oxidation of IMP to XMP proceeds through a covalent mechanism involving an active site cysteine residue. This enzyme is a target for immunosuppressive agents because it catalyzes a key step in purine nucleotide biosynthesis, which is important for the proliferation of lymphocytes (12,13). Since an inhibitory effect of inosine on IMPDH may have provided a mode for some of inosine's anti-inflammatory actions, we have tested whether inosine affects the activity of recombinant IMPDH. The uncompetitive IMPDH inhibitor MPA, the active metabolite of the immunosuppressive agent mycophenolate mofetil, was used as a positive control. The findings clearly show that inosine does not affect the activity of IMPDH, and therefore this pathway is not involved in its biological actions.

Which mechanisms, then, are responsible for the *in vivo* anti-inflammatory effects of inosine? Our recent studies suggest that the effect of inosine is not sensitive to nucleoside uptake into cells and is partially mediated via adenosine receptors. First, we studied, whether cellular uptake of inosine is necessary for its effect. To this end, we used dipyridamole, a selective blocker of nucleoside uptake, in murine peritoneal macrophages (the production of TNF- α being measured as an indicator). Although dipyridamole alone caused a substantial inhibition of TNF- α release, dipyridamole and inosine together additively suppressed the production of this cytokine suggesting that the effect of inosine cannot be prevented by blockade of nucleoside uptake (3). Subsequent studies demonstrated that inosine fails to alter LPS-induced I κ B degradation, MAPK, and JNK activation. The p42/44 MAPK, p38 MAPK, and JNK are important intracellular components of the inflammatory responses to LPS (3). Inosine also failed to affect intracellular cyclic AMP levels (3). Using RNase protection assay, we also examined whether inosine can modify the expression of a host of cytokine mRNAs in LPS stimulated

macrophages. Although LPS induced a strong increase of mRNA levels of TNF- α , MIP-1 α , MIP-2, RANTES, and TGF- β , inosine failed to suppress this response (3). Therefore, the effect of inosine on cytokine production is most likely post-transcriptional. This post-transcriptional inhibitory effect may explain the suppression of cytokine production during shock induced by endotoxin and cecal ligation and puncture (5).

Because of structural similarities to adenosine, we surmised that the effect of inosine was mediated by an adenosine receptor. Both A₁ and A₂ antagonists alone augmented TNF- α production, suggesting that endogenous adenosine inhibits cytokine production. However, in the presence of both antagonists the inhibition by inosine of TNF- α was significantly, although not completely, abrogated, suggesting that the effect of inosine is, at least partially mediated via adenosine receptors (3). The effect of inosine was suppressed, but not abolished in peritoneal macrophages lacking A_{2a} receptors, indicating the partial involvement of this receptor subtype. Because inosine is degraded to hypoxanthine in murine peritoneal cells, we examined if the effect of inosine is mediated by hypoxanthine. However, in contrast to inosine, hypoxanthine failed to alter TNF- α release (3). Various approaches of adenosine receptor down-regulation have also demonstrated that inosine may exert some of its effects via down-regulation of adenosine receptors (3).

Poly(ADP-ribose) polymerase (PARP), also known as poly(ADP-ribose) synthetase or PARS, is an abundant nuclear enzyme of eukaryotic cells, which has been implicated in response to DNA injury and oxidant-induced cell death (23,24). PARP is a nuclear nick sensor enzyme that becomes activated in response to DNA damage. Activated PARP cleaves NAD⁺ into nicotinamide and ADP-ribose and triggers necrotic cell death. PARP has also been implicated in the transcriptional regulation of inflammatory mediators: PARP activation triggers the expression of TNF- α and a variety of other inflammatory mediators *in vitro* and *in vivo* (23,24). We recently examined whether inosine and other purines may also exert additional effects by interfering with the PARP activation pathway. Similar to the working hypothesis in the case of IMPDH (see above), our assumption was based on the structural similarity of hypoxanthine, inosine and adenosine to part of NAD, the substrate of PARP. The results (25) demonstrated that inosine dose-dependently inhibit PARP activation in macrophages. Based on the concentrations necessary for PARP inhibition, and the therapeutic doses of inosine, it is conceivable that PARP inhibition also contributes to the anti-inflammatory effects of this purine.

The breakdown of inosine yields urate. Urate is an important endogenous antioxidant, a scavenger of oxyradicals and of peroxynitrite (26-29). Uric acid administration has been shown to exert protective effects in animal models of shock (28) and neuroinflammation (29). Therefore, a final, potential mechanism whereby inosine can exert beneficial effects *in vivo* is by enhancing endogenous urate levels. It is conceivable that some of the suppression by inosine of cytochrome C reduction by activated neutrophils may be related to rapid conversion of inosine to urate, followed by direct superoxide scavenging.

Similarly to the previous studies, in the current study, inosine exerted its *in vitro* effects in the low millimolar concentrations. Although this is consistent with its *in vivo*

potency (several hundreds mg per kg body weight oral or intraperitoneal dosing), this concentration range is outside of what many investigators consider physiologically or therapeutically relevant. Also, inosine is clearly much less potent than its parent compound, adenosine. This low potency of inosine is the likely reason why previous studies failed to note some of the effects reported here. For instance, in previous studies inosine was tested in the dose range of 1-100 μ M in human neutrophils, and no effect was reported (30,31), a finding, which is consistent with the current results. When interpreting the current findings, one has to keep in mind, however, that the doses of inosine safely tolerated by living beings are massive. For example, inosine has been given to humans up to 10 g/day dosing, and no adverse effects were reported; in fact inosine is widely available as a nutritional supplement in health food stores (32-34). An effect seen with inosine in the low millimolar range, which may be dismissed or considered negligible when thinking about a classical receptor ligand or enzyme inhibitor drug, may nevertheless be reasonable and therapeutically achievable in the case of inosine. Considering the established safety profile of inosine in humans, coupled with its anti-inflammatory effects in human cells (present study) as well as in rodent models *in vivo* (3-6), and its effects in some of the human *in vitro* assays used in the present study, further testing of inosine in humans may be justified.

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