ATP stimulates the secretion of caspase-1 from human peripheral blood cells through a mechanism involving the purinergic receptor P2X7

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ABSTRACT
The mechanisms involved in chronic pain and inflammation have come under intense scrutiny in the last two decades. With non-steroidal anti-inflammatory drugs (NSAIDs) providing suboptimal relief for inflammatory pain, the targeting of other pro-inflammatory pathways is being investigated for therapeutic application. The purinergic receptor P2X7 is thought to regulate the activation of caspase-1, which subsequently cleaves pro-interleukin-1β (IL-1β), thereby causing the secretion of mature IL-1β. In this study, we utilized an ex vivo stimulation of human peripheral blood with variable duration lipopolysaccharide (LPS) priming and examined the effects of ATP, P2X7 antagonists, and NSAIDS on IL-1β and caspase-1. We observed that following LPS priming, the P2X7 agonist and ATP analogue BzATP markedly stimulated IL-1β secretion. Interestingly, BzATP also stimulated the secretion of caspase-1 itself in a dose and time-dependent manner that paralleled the secretion of IL-1β, which was also dependent upon LPS priming. The secretion of both IL-1β and caspase-1 was almost completely blocked by the P2X7 antagonists PPADS and AZ11645373. Moreover, secretion of both IL-1β and caspase-1 was inhibited by AZ11645373 in a parallel, dose-dependent manner. In contrast, NSAIDS were unable to inhibit secretion of either IL-1β or caspase-1. These results suggest that both ATP-induced IL-1β secretion and caspase-1 secretion are mediated almost entirely through the P2X7 receptor. These data also further indicate that by inhibiting IL-1β and caspase-1 secretion, P2X7 antagonism may represent a novel mechanism to inhibit pain and inflammation that is not achievable with currently available NSAIDS.

KEYWORDS: P2X7, caspase-1, interleukin-1β, purinergic receptor, non-steroidal anti-inflammatory drugs, cyclo-oxygenase

INTRODUCTION
Over the past two decades, the mechanisms of chronic pain and inflammation have been investigated extensively. While inflammation has been known to be a key component of diseases such as rheumatoid arthritis and asthma, it has also begun to be implicated in the pathophysiology of many other diseases including Alzheimer’s disease, diabetes, and depression [1-2]. In addition, inflammatory processes are now thought to be directly associated with the development and maintenance of pain [3-9].

Currently, the most common treatment for pain is the use of nonsteroidal anti-inflammatory drugs (NSAIDS), which are taken by about 50 million Americans a day [10]. NSAIDS target the cyclo-oxygenase (COX) pathway. However, these
medications commonly can lead to gastrointestinal and renal side-effects [10]. In addition, NSAIDS are not effective in treating all types of pain. As a result, there has been a continued search to develop new pain medications that focus on the upstream regulators of COX.

Interleukin-1 beta (IL-1β) is one of these upstream regulators that has received substantial attention [3]. Produced by myeloid cells, tissue macrophages, dendritic cells, and peripheral blood mononuclear cells, this cytokine is quickly released upon exposure to danger-associated pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs) [11]. Originally synthesized as a precursor protein [12], once cleaved and secreted, mature IL-1β activates the transcription of numerous pro-inflammatory genes which can lead to increased reactive oxygen species, nitric oxide synthesis, and COX activity [13-14].

In 1989, Kostura and colleagues identified an enzyme which converted pro-IL-1β to its mature form [15]. This convertase, now called caspase-1, is constitutively expressed as an inactive 45 kD zymogen in monocytic cells [16]. For caspase-1 to be activated, the NOD-like receptor P3 (NLRP3) inflammasome must be assembled. Its formation clusters pro-caspase-1 molecules, triggers their autocatalysis, and subsequently results in the formation of the activated p10/p20 caspase tetramer [17]. Because caspase-1 deficient mice have been demonstrated to have decreased nociception to inflammatory pain, understanding the role that caspase-1 plays in pain in humans has become increasingly important [3, 18].

The purinergic receptor P2X7 is believed to activate the NLRP3 inflammasome [17]. P2X7 is an ATP-gated cation-selective channel permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\). Many investigations of P2X7 have utilized animal models to examine the effects of P2X7 antagonism and to characterize this receptor channel. Use of these models has shown that antagonism of P2X7 can reduce hypersensitivity to pain [19]. Using whole blood stimulation, Al-Shukaili and coworkers discovered that compared to healthy controls, IL-1β production but not P2X7 expression was increased in peripheral blood mononuclear cells from individuals suffering from rheumatoid arthritis [20]. In light of these findings, we sought to further characterize P2X7 regulation of IL-1β and caspase-1. In this study, we demonstrate that ATP dose-dependently stimulates not only the secretion of IL-1β from human peripheral blood but also the secretion of caspase-1 itself. We also demonstrate that ATP-stimulated IL-1β release and caspase-1 secretion are mediated almost entirely through P2X7. Finally, we show that NSAIDS are ineffective at preventing either ATP-induced IL-1β or caspase-1 release, further suggesting that P2X7 antagonism may represent a novel mechanism for the treatment of pain and inflammation.

**METHODS**

**Collection of venous blood**

Peripheral blood was collected from volunteer donors who each gave informed consent for their blood to be collected for this study. Donors were requested to stop using NSAIDS and glucocorticoids at least 5 days prior to their donation. Venous blood was drawn into 10 mL Vacutainer® Heparin Plasma Tubes, (Becton, Dickinson, and Company). Tubes were inverted slowly several times to disperse the anticoagulant and stored overnight at room temperature, prior to ex vivo stimulation the following day.

**LPS stimulation**

All blood from each donor was pooled and gently mixed in a 50 mL conical tube. One mL of blood was then added to a 15-mL polypropylene round-bottom tube (BD Falcon). The blood was subsequently diluted with 1 mL of RPMI 1640, which had been warmed to 37°C before its addition. Lipopolysaccharide (LPS) from E. coli, serotype 055:B5, (Enzo Life Sciences) was dissolved to a concentration of 1 mg/mL using HyPure water (ThermoScientific). LPS was subsequently dissolved in a solution of RPMI 1640 + 1% BSA to a concentration of 0.2 µg/mL and added to each tube to achieve a final LPS concentration of 2 ng/mL. Next, tubes were loosely capped and placed on a plate shaker inside a humidified 37°C cell culture incubator containing 95% air and 5% CO\(_2\) for 0-90 minutes. After each incubation, tubes were centrifuged at 800 x g for 15 minutes to pellet the cells. Supernatants were removed and stored at -80°C prior to subsequent analysis of IL-1β and caspase-1 concentrations.
ATP stimulates caspase-1 secretion through P2X7 dependent mechanism

Addition of NSAIDS, P2X7 antagonists, and BzATP

NSAIDS, P2X7 antagonists, and BzATP were all obtained from Sigma Aldrich and used at the following concentrations: 900 µM PPADS (Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate), 11 µM AZ11645373, 20 µM Acetaminophen (4’-Hydroxy-acetanilide), 4-Acetamidophenol, N-Acetyl-4aminophenol), 20 µM Aspirin (Acetylsalicylic acid), 20 µM Ibuprofen (α-Methyl-4-(isobutyl)phenylacetic acid), 20 µM Naproxen ((S)-(−)-2-(6-Methoxy-2-naphthyl)propionic acid), and 10 µM Celecoxib (4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene sulfonamide). NSAIDS and P2X7 antagonists were added at their desired concentrations prior to diluting the blood with RPMI 1640. The tubes were loosely capped and placed inside a humidified 37°C cell culture incubator for 2 hrs under an atmosphere of 95% air and 5% CO₂. After priming with LPS for 0-90 minutes, blood was stimulated with various concentrations of 2’-3’-o-(4-benzoylbenzoyl)-adenosine-5’-triphosphate triethylammonium salt (BzATP) for an additional 30 minutes.

Measurements of IL-1β and caspase-1

Stored samples were brought to room temperature, vortexed, and centrifuged for 10 min at 10,000 x g. Supernatants were decanted, and IL-1β concentrations were determined using a human IL-1 beta ELISA (R&D Systems). Caspase-1 concentrations were measured using a human Caspase-1 ELISA (R&D Systems). All analyses were performed according to the manufacturer’s instructions.

Statistical analysis

All data were expressed as the mean ± SEM. Statistical significance was determined using Microsoft Excel to calculate a one-tailed Student’s t-test with unequal variance. A p-value of <0.05 was considered to indicate statistical significance.

RESULTS

We first characterized the effect of LPS exposure prior to ATP-induced IL-1β release. We primed peripheral blood with 2 ng/mL LPS for 0-90 minutes prior to the addition of 600 µM BzATP to the samples for an additional 30-minute incubation. Figure 1A shows the effect of LPS priming duration on the secretion of IL-1β. With no exposure to LPS, 5 ± 2 pg/mL of IL-1β was released. As the duration of LPS priming lengthened, however, there was a dose-dependent increase in the secretion of IL-1β. After 30, 60 and 90 minutes of LPS priming, BzATP-induced IL-1β release had increased to 103 ± 10 pg/mL, 322 ± 41 pg/mL, and 1888 ± 268 pg/mL respectively (p = 0.001, 0.002, 0.003 respectively, compared to baseline).

We next investigated whether caspase-1 itself might be similarly secreted and if so how changes in ATP-induced caspase-1 secretion might also be influenced by the duration of LPS priming. Figure 1B shows the results from this series of experiments, in which caspase-1 secretion followed a similar trend to that of IL-1β. With no exposure to LPS, 96 ± 5 pg/mL of caspase-1 was released. Again, as the duration of LPS priming lengthened, there was a dose-dependent increase in the BzATP-induced secretion of caspase-1. After 30, 60 and 90 minutes of LPS priming, BzATP-induced caspase-1 release had increased to 232 ± 47 pg/mL, 390 ± 47 pg/mL and 490 ± 84 pg/mL respectively (p = 0.02, 0.007, 0.002, respectively, compared to baseline).

Based on these data, the duration of LPS priming was determined to be an important influence on both ATP-stimulated IL-1β and caspase-1 secretion, with maximal secretion of both observed after 90 minutes of LPS priming. Therefore, we further investigated the effect of various concentrations of BzATP on IL-1β secretion following 90 minutes of LPS priming. Figure 2A shows the results from this series of experiments in which BzATP stimulated IL-1β secretion in a dose-dependent manner. With no BzATP added, baseline IL-1β secretion was 158 ± 63 pg/mL. Addition of 300 µM BzATP only modestly increased IL-1β release to 303 ± 129 pg/mL (p = 0.173 compared to baseline). Addition of 600 µM BzATP, however, markedly stimulated IL-1β release (4191 ± 1230 pg/mL, p = 0.011 versus baseline). At concentrations of 900 - 2,400 µM BzATP, comparable levels of IL-1β were
ATP-induced secretion of both IL-1β and caspase-1 is increased as a function of duration of LPS priming. Peripheral venous blood from volunteer donors was primed with 2 ng/mL LPS for 0-90 minutes prior to the addition of 600 µM BzATP for an additional 30 minutes. At the end of the incubation, the amount of secreted IL-1β was measured. Data are shown as the mean ± SEM (n = 6 donors from 2 independent experiments).

Figure 1B. ATP-induced secretion of both IL-1β and caspase-1 is increased as a function of duration of LPS priming. Samples from Figure 1A were analyzed for secreted caspase-1. Data are shown as the mean ± SEM (n = 6 donors from 2 independent experiments).
ATP stimulates caspase-1 secretion through P2X7 dependent mechanism

Figure 2A. ATP stimulates both IL-1β and caspase-1 secretion in a dose-dependent manner. Peripheral blood was primed with 2 ng/mL LPS for 90 minutes prior to the addition of 0-2,400 µM BzATP for an additional 30 minutes. At the end of the incubation, the amount of secreted IL-1β was measured. Data are shown as the mean ± SEM (n = 6 donors from 2 independent experiments).

Figure 2B. ATP stimulates both IL-1β and caspase-1 secretion in a dose-dependent manner. Samples from Figure 2A were analyzed for secreted caspase-1. Data are shown as the mean ± SEM (n = 6 donors from 2 independent experiments).

released compared to those observed after stimulation with 600 µM BzATP (p = 0.002, 0.005, 0.005, 0.003 respectively, compared to baseline), with a maximum level of 5571 ± 1059 pg/mL observed after addition of 900 µM BzATP.

Following these experiments, we next investigated the effect of various concentrations of BzATP on caspase-1 secretion after 90 minutes of LPS priming. Figure 2B shows the results from these experiments in which BzATP was also shown to stimulate caspase-1 secretion in a dose-dependent manner similar to that of IL-1β. With no BzATP added, baseline caspase-1 secretion was 163 ± 30 pg/mL. Addition of 300 µM BzATP only
modestly increased caspase-1 release to 174 ± 35 pg/mL ($p = 0.21$ compared to baseline). Addition of 600 µM BzATP, however, markedly stimulated caspase-1 release (388 ± 111 pg/mL, $p = 0.05$ versus baseline). At concentrations of 900 - 2,400 µM BzATP, increasing levels of caspase-1 were released compared to those observed after stimulation with 600 µM BzATP ($p = 0.0008$, 0.001, 0.002, 0.0006 respectively compared to baseline), with a maximum level of caspase-1 release of 637 ± 84 pg/mL observed with 900 µM BzATP.

After obtaining these results, we investigated the effects of the P2X7 antagonists PPADS and AZ11645373 on Bz-ATP induced IL-1β and caspase-1 secretion. In this series of experiments, peripheral blood was incubated with P2X7 antagonists for 2 hours and primed with 2 ng/mL LPS for 90 minutes before being exposed to 900 µM BzATP for an additional 30 min. Figure 3A shows the effect of these P2X7 antagonists on IL-1β secretion as a percent inhibition in relation to the no-antagonist control. The P2X7 specific antagonists PPADS and AZ11645373 almost completely abolished the BzATP-induced IL-1β release with 99.1 ± 0.3% and 95.7 ± 0.8% inhibition observed respectively (both $p = 0.0003$ compared to control). Figure 3B demonstrates the effect of these P2X7 antagonists on caspase-1 secretion as a percent inhibition in relation to the no antagonist control. The P2X7 specific antagonists PPADS and AZ11645373 largely blocked the BzATP-induced caspase-1 release with 76.8 ± 12.01% and 87.1 ± 5.9% inhibition observed respectively (both $p = 0.002$ compared to control).

To further investigate the P2X7 dependence of caspase-1 and IL-1β secretion, we stimulated peripheral blood from 6 donors with increasing concentrations of the antagonist AZ11645373. Figure 4 shows the effect of increasing concentrations of AZ11645373 on IL-1β and caspase-1 secretion as a percent inhibition in relation to the no-antagonist control. Maximal inhibition of secreted IL-1β (95.91 ± 1.07%, $p = 0.003$) and of secreted caspase-1 (93.96 ± 4.52%, $p = 0.001$) was achieved using 10.7 µM AZ11645373. Although the inhibition dose-response continues into nanomolar concentrations of AZ11645373, the inhibition responses remained statistically significant at 2 µM AZ11645373, at which concentration IL-1β secretion was inhibited by 65.71 ± 6.72% and caspase-1 secretion was inhibited by 76.73 ± 10.89% ($p = 0.01$ and $p = 0.002$, respectively).

Lastly, we examined the influence of commonly prescribed NSAIDS on IL-1β and caspase-1 secretion. In these experiments, peripheral blood was incubated with NSAIDS for 2 hours and primed with 2 ng/mL LPS for 90 minutes before being exposed to 900 µM BzATP for an additional 30 min. The NSAIDS used during these experiments included 20 µM Acetaminophen, 20 µM Aspirin, 20 µM Ibuprofen, 20 µM Naproxen, and 10 µM Celecoxib. As Figures 3A and 3B demonstrate, none of these commonly prescribed NSAIDS significantly inhibited BzATP-induced IL-1β or caspase-1 release.

**DISCUSSION**

The purinergic receptor P2X7 is involved in the activation of the inflammasome, the subsequent maturation of caspase-1, and the resultant secretion of IL-1β. In this study, we utilized LPS priming followed by BzATP stimulation to characterize the secretion of not only IL-1β but also of caspase-1 itself in human peripheral blood. We demonstrated that BzATP induced secretion of IL-1β and of caspase-1 was almost completely inhibited with P2X7 antagonists. In addition, we observed that none of the commonly prescribed NSAIDS caused any inhibition of ATP-induced secretion of either IL-1β or caspase-1. Our data expand upon the findings of Ferrari and colleagues by demonstrating the P2X7 specificity of IL-1β secretion in whole blood [21]. In addition, we also observed that the secretion of mature caspase-1 is influenced by the duration of LPS exposure prior to P2X7 activation and by the concentration of the P2X7 agonist BzATP.

LPS is commonly used to up-regulate pro-IL-1β synthesis via Toll-like Receptor (TLR) [4]. In doing so, LPS mimics the role of TLR induction in chronic inflammatory states [22]. Several studies have shown that LPS can activate caspase-1 and promote the secretion of IL-1β, possibly through autocrine or paracrine release of ATP and subsequent induction of P2X7 [23-26]. In most
Figure 3A. BzATP induced secretion of IL-1β and of caspase-1 is almost completely inhibited by P2X7 antagonists; and neither IL-1β nor caspase-1 release is inhibited by NSAIDS. Peripheral blood was incubated with P2X7 antagonists or NSAIDS for 2 hrs before being primed with 2 ng/mL LPS for 90 minutes prior to the addition 900 µM BzATP for an additional 30 minutes. At the end of the incubation, the amount of secreted IL-1β was measured. Data are expressed as percent inhibition compared to control and are shown as the mean ± SEM (n = 6 donors from 2 independent experiments).

Figure 3B. BzATP induced secretion of IL-1β and of caspase-1 is almost completely inhibited by P2X7 antagonists; and neither IL-1β nor caspase-1 release is inhibited by NSAIDS. Samples from Figure 3A were analyzed for secreted caspase-1. Data are expressed as percent inhibition compared to control and are shown as the mean ± SEM (n = 6 donors from 2 independent experiments).
The P2X7 activity responsible for the secretion of IL-1β is modulated by the concentration of agonist in the surrounding environment. Despite the amount of pro-IL-1β in the cytoplasm and the catalytic activity of caspase-1 remaining constant, secreted IL-1β increases in response to P2X7 agonist concentration [25-26, 28]. It is therefore conceivable that increased concentrations of P2X7 agonists result in either a larger amount of caspase-1 being activated or an enhancement of the secretory pathway utilized by IL-1β [28]. This idea is supported by Yan and colleagues, who demonstrated that the kinetics of P2X7 are dependent on the number of occupied binding sites [29].

At higher concentrations of P2X7 agonist, activity is shifted from cation exchange to cellular pores permeable to molecules as large as 900 Da [30-31]. The formation of these pores is believed to be dependent on concentration and duration of exposure to ATP [31-33]. Thus, in our experiments, it is possible that above 900 µM BzATP, formation of this pore occurs so rapidly that caspase-1 secretion does not further increase.
Likewise, the plateau of IL-1β secretion observed with concentrations of 900 µM BzATP or greater may also be related to the rate kinetics of caspase-1 activation and consequent IL-1β processing prior the rapid-formation of the non-selective pore.

The P2X7 specificity of ATP-induced IL-1β and caspase-1 secretion was examined by using the commercial P2X7 antagonists PPADS and AZ11645373 [34]. Both P2X7 antagonists abolished IL-1β and caspase-1 secretion. To further scrutinize P2X7 regulated secretion of IL-1β and caspase-1, a dose response to AZ11645373 was established. The data generated suggested that secretion of both molecules is directly regulated by P2X7 in a similar manner. This suggests that possibly due to the kinetics of P2X7 pore-opening and regardless of being constitutively expressed (caspase-1) or having to have precursor synthesis upregulated (IL-1β), both caspase-1 and IL-1β secretion are dependent on P2X7 activity. It is understandable that a change in the amount of activated caspase-1 would directly correlate to how much mature IL-1β is secreted. It is interesting to note, however, that inhibition of caspase-1 secretion parallels that of secreted IL-1β using AZ11645373. This suggests that secretion of both molecules is directly regulated by P2X7 during states of inflammation.

BzATP is approximately 10-fold more potent than ATP [35]. Using a conservative calculation, the concentration of ATP equivalent to that of BzATP used in our experiments would be in the low millimolar range. During injury or inflammation, local extracellular ATP concentrations can rise to these levels due to exocytosis or lysis, resulting in the release of cellular cytoplasmic concentrations of ATP [36-38]. Thus, in a localized environment, it is not inconceivable that millimolar levels of ATP could be achieved for sustained for P2X7 activation.

While the effects of secreted IL-1β are well characterized, the consequences of secreted caspase-1 are not. It is possible that extracellular caspase-1 remains proteolytically active, as was reported for caspase-3 [39]. If this is the case, then caspase-1 may still function to cleave immature IL-1β that was secreted from surrounding cells and perpetuate the inflammatory response. Cowan and colleagues recently reported that caspase-2, previously known to have only one intracellular substrate, can also degrade elastin, a component of the extracellular matrix [40]. In addition, Laliberte and colleagues reported that caspase-1 could be externalized from isolated human monocytes treated with ATP via an ethacrynic acid-sensitive process. However, this study did not determine the effect of the duration of LPS priming, the dose-dependency of the response, or if the mechanism involved P2X7 [41]. Thus, it may be possible that secreted caspase-1 could also degrade the extracellular matrix, further contributing to localized inflammation.

Because currently prescribed medications such as NSAIDS are still far from optimal for the treatment of pain and inflammation, continued efforts will be made to explore potential novel mechanisms mediating pain and inflammation such as P2X7. Our findings in this study shed light onto the biology of ATP mediated IL-1β secretion in human peripheral blood by demonstrating that ATP dose-dependently increases the secretion of IL-1β in a manner that is almost completely mediated through P2X7. In addition, we also found that the secretion of caspase-1 itself was dose-dependently stimulated by ATP and mediated by P2X7. Importantly, none of the currently available NSAIDS were observed to inhibit ATP-induced secretion of either IL-1β or caspase-1. These data suggest that P2X7 antagonist mediated inhibition of IL-1β and caspase-1 release may provide a mechanism to treat pain and inflammation that is not addressed by administration of NSAIDS.

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REFERENCES