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A putative osmoreceptor system that controls neutrophil function through the release of ATP, its conversion to adenosine, and activation of A2 adenosine and P2 receptors

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Abstract: We have previously shown that hypertonic stress (HS) can suppress chemoattractant-induced neutrophil responses via cyclic adenosine monophosphate and enhance these responses through p38 mitogen-activated protein kinase (MAPK) activation. The underlying mechanisms are unknown. Here, we report that HS dose-dependently releases adenosine 5′-triphosphate (ATP) from neutrophils and that extracellular ATP is rapidly converted to adenosine or activates p38 MAPK and enhances N-formyl-methionyl-leucyl-phenylalanine-induced superoxide formation. In contrast, adenosine suppresses superoxide formation. Adenosine deaminase treatment abolished the suppressive effect of HS, indicating that HS inhibits neutrophils through adenosine generation. Neutrophils express mRNA, encoding all known P1 adenosine receptors (A1, A2a, A2b, and A3) and the nucleotide receptors P2Y2, P2Y4, P2Y6, P2Y11, and P2X7. A2 receptor agonists mimicked the suppressive effects of HS; the A2 receptor antagonists 8-(p-sulfophenyl)theophylline, 3,7-dimethyl-1-(2-propynyl)xanthine, 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine, and 3-propylxanthine, but not A1 and A3 receptor antagonists, decreased the suppressive effect of HS, indicating that HS suppresses neutrophils via A2 receptor activation. Antagonists of P2 receptors counteracted the enhancing effects of ATP, suggesting that HS costimulates neutrophils by means of P2 receptor activation. We conclude that hypertonic stress regulates neutrophil function via a single molecule (ATP) and its metabolite (adenosine), using positive- and negative-feedback mechanisms through the activation of P2 and A2 receptors, respectively. *J. Leukoc. Biol.* 76: 245–253; 2004.

Key Words: *phagocyte · hyperosmotic stress · oxidative burst · inflammation · purinergic receptors*

INTRODUCTION

Polymorphonuclear neutrophils (PMN) form the first line of defense against invading microorganisms and play a critical role in the inflammatory response of many diseases. Activation of PMN promotes chemotaxis, phagocytosis, the release of stored enzymes, and an oxidative burst that produces reactive oxygen species including the superoxide radical (O_2^-) [1]. However, PMN activation can be a double-edged sword. PMN not only protect the host from invading microorganisms, but they are also involved in the pathogenesis of acute inflammatory diseases. For example, following traumatic injury, excessively activated PMN release cytotoxic mediators that damage host tissues, resulting in serious complications, such as acute respiratory distress and multiple organ failure syndromes. The pathophysiological roles of PMN in tissue damage following inflammatory responses after trauma imply that blunting of PMN activation may reduce post-traumatic complications.

Infusion of hypertonic saline (HS) is a novel therapeutic approach that can be used to reduce post-traumatic complications [2]. HS, with or without the addition of colloids such as dextran or hetastarch, can be used to resuscitate patients from hemorrhagic shock. As only small volumes (e.g., 250 ml) of such fluids are sufficient to restore circulation, initial interest in HS was primarily based on the logistical benefits of such treatment. Later, however, we found that HS resuscitation can prevent neutrophil activation in a mouse model of hemorrhagic shock [3]. Parallel results were obtained in a clinical trial in which HS resuscitation improved the outcome in patients, apparently by reducing neutrophil-mediated, post-traumatic complications [4]. The notion that HS infusion can prevent PMN activation has been confirmed by additional studies in which HS resuscitation protected host tissues from PMN-mediated damage [5].

Infusion of HS in patients increases plasma tonicity to levels that can suppress or enhance different PMN functions, including superoxide formation [6–9]. These levels of HS activate signaling pathways that are analogous to those involved in the response of yeast to hypertonic stress [10]. Although yeast cells have at least two known osmoreceptors that regulate responses to osmotic changes in their environment [10], the molecular

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mechanisms by which PMN and other mammalian cells detect osmotic stress are poorly understood [11].

We have previously reported that HS can enhance or suppress PMN functions depending on the timing of hypertonic stimulation relative to other stimuli. Enhancement is mediated by activation of the p38 mitogen-activated protein kinase (p38 MAPK), and suppression occurs via increases in cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA), which blocks N-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor signals that promote functions such as the oxidative burst [8, 9]. However, the mechanisms by which HS exerts these opposing effects are unclear. We show here that HS-induced cell shrinkage releases adenosine 5′-triphosphate (ATP) from PMN, released ATP augments PMN functions through P2 receptors and p38 MAPK activation, or ATP is converted to adenosine, which suppresses PMN functions via A2 receptors that activate cAMP/PKA signaling. This bidirectional control by released ATP thus allows PMN to register and differentially respond to osmotic changes in their extracellular environment.

MATERIALS AND METHODS

Materials

8-(p-Sulfophenyl)theophylline (8-SPT), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC), 3,7-dimethyl-1-(2-propynyl)xanthine (DMPX), 3-propylxanthine (enprofylline), 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191), N^6-cyclopentyladenosine (CPA), 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrobromide-hemicarbonate salt (CGS-21680), and 1-deoxy-1-(6-[([3-Iodophenyl]methyl) amino]-9H-purin-9-yl)-N-methyl-β-D-ribofuranuronamide (IB-MECA) were from Sigma-Aldrich Co. (St. Louis, MO). Periodate-oxidized ATP (o-ATP), 1-[N, O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), and suramin were from Calbiochem (San Diego, CA). Dextran 500 and Percoll were from Pharmacia (Piscataway, NJ).

Cell isolation

PMN were isolated from peripheral blood of healthy human volunteers and were stimulated exactly as described previously [8]. Briefly, heparinized blood was mixed at a ratio of 3:1 with a dextran solution (5% w/v) in pyrogen-free, normal saline and was allowed to sediment for 30 min at room temperature. Cells in the supernatant were centrifuged, resuspended in Hanks' buffered saline solution (HBSS), and separated with Percoll gradient centrifugation according to the manufacturer's recommendations. The purified PMN preparation was washed twice with HBSS. Viability was >95%, as assessed with trypan blue dye exclusion, and purity exceeded 98%. This isolation procedure was chosen to avoid osmotic shock lysis that is often used to remove residual red blood cells. Cell isolation and all subsequent experiments were performed under sterile and pyrogen-free conditions.

Cell stimulation

Cells were preincubated at 37°C for 1 h before stimulation as described in the experiments below. Cells were stimulated with hypertonic saline by the addition to the culture medium of appropriate volumes of HBSS that contained an additional 1 M NaCl.

Assay of neutrophil function

O_2^- formation was determined with a slightly modified version of the cytochrome C reduction assay described previously [9]. Briefly, PMN (2×10^6/ml) were incubated at 37°C and treated with the different reagents as described in the experiments below, then 100 μl cytochrome C (2 mM) was added, and the cells were stimulated with 100 nM fMLP for 10 min, placed on ice, and centrifuged in the cold. The optical density at 550 nm of the supernatants was used to calculate O_2^- formation. As a control, superoxide dismutase (50 μg/ml) was added to verify that cytochrome C reduction was the result of O_2^- formation. Depending on the different cell preparations and blood donors, superoxide formation could range from 36 to 98 nmol/min/2×10^8 cells in response to 100 nM fMLP and from 0 to 6 nmol/min/2×10^8 cells in unstimulated cells. Unless otherwise indicated, changes of superoxide formation in response to HS and other modulatory agents were expressed as percentage of the full response to 100 mM fMLP.

High-performance liquid chromatography (HPLC)

Freshly isolated PMN (250 μl at 10^7/ml) were stimulated with HS as described below for the different experiments and placed on ice, and PMN suspensions were centrifuged in the cold (325 g, 1 min). The supernatants (200 μl) were processed for analysis by addition of 1/20 vol ice-cold 8 M perchloric acid, centrifugation at 10,000 *g* for 5 min, adjustment of pH to 6 with 4 M K_2HPO_4, and another centrifugation. Then, 25 μl of these samples was subjected to HPLC analysis using a Waters system (Waters Associates, Milford, MA) consisting of two 510 pumps, a U6K injector, a tunable Waters 484 UV absorbance detector set at 254 nm, and a NEC computer system with Baseline 810 chromatography software. Ion-pair separation of nucleotides was achieved with a Supelcosil LC-18-T reverse-phase column (C18, 15 cm×4.6 mm, 3 μm particles) and Supelguard LC-18-T guard column (Supelco, Sigma-Aldrich Co.). Chromatographic conditions were as follows: buffers A (0.1 M KH_2PO_4, 4 mM tetrabutylammonium hydrogen sulfate, pH 6) and B (buffer A:methanol, 70:30); gradient: 0–2.5 min, 100% A; 2.5–5 min, 0–20% B; 5–10 min, 20–40% B; 10–13 min, 40–100% B; 13–18 min, 100% B; 18–18.1 min, 100–0% B; 18–22 min, 100% A; flow rate: 1.5 ml/min.

Purinergic receptor mRNA levels

Transcription of mRNA encoding the four adenosine receptors and the different P2 receptors was examined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from human neutrophils using Qiagen RNeasy RNA kit (Qiagen, Studio City, CA). The real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen) following the manufacturer's protocol. Total RNA (5 ng) was used as template, and primer concentration was 0.5 μM. An initial activation step of 15 min (95°C) was performed before 49 cycles of the following: 30 s (94°C), 30 s (58°C), and 40 s (72°C). For the adenosine receptors, we used the following primer pairs: A1 (305 bp, 5′-GCCACAGACCTACTTCCACA-3′ and 5′-CCTTCTCGAACTCGCACTTG-3′); A2a (216 bp, 5′-CGAATTCAACCTGCAGAACGTCACC-3′ and 5′-TCGAATTCGCGGTCAATGGCGATG-3′); A2b (513 bp, 5′-CAGACGCCCACCAACTACTT-3′ and 5′-GCCACCAGGAAAATCTTAATG-3′); A3 (328 bp, 5′-ACCACTCACAGAAGAATATG-3′ and 5′-ACTTAGCCGTCTTGAACTCC-3′). These and the P2 receptor primer sequences and PCR conditions were based on previously published work [12–14].

Purinergic receptor expression

Adenosine receptors were visualized by fluorescence microscopy as described before [15] except that PMN were treated with 0.25 mM diisopropyl fluorophosphate (Sigma-Aldrich Co.) at 0°C for 30 min and fixed with 4% paraformaldehyde in phosphate-buffered saline at room temperature for 15 min. To allow antibodies to bind to transmembrane and intracellular receptor epitopes, cells were permeabilized with 0.5% Triton X-100 for 1 min on ice before staining with 20 μg/ml antibodies to A1, A2a, A2b, A3, P2X7 (Santa Cruz Biotechnology, Santa Cruz, CA), or P2Y2 (Zymed Laboratories, South San Francisco, CA) at room temperature. Control cells were treated identically to the antibody-stained cells, except that both antibodies were omitted, or the primary antibody was replaced by unrelated immunoglobulin G.

Intracellular cAMP measurements

Neutrophil cAMP levels were determined with an immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ) as described before [9]. Briefly, neutrophils (10^6 cells) were plated in 96-well tissue-culture plates and incubated at 37°C for 1 h. Cells were treated with receptor antagonists for 30 min, 3-isobutyl-1-methylxanthine (IBMX) was added at a final concentration of 80 μM, and

cells were incubated with 20 mM HS for 10 min at 37°C. Then 20 μl of the lysis reagent supplied with the enzyme immunoassay kit was added, and cAMP levels were determined according to the manufacturer's instructions.

Measurement of MAPK activation

To measure p38 and extracellular-regulated kinase (ERK) MAPK activation, PMN (10^6 cells/sample), stimulated as described below, were placed on ice, centrifuged, resuspended in 100 μl ice-cold sodium dodecyl sulfate sample buffer containing 100 mM dithiothreitol, and lysed by boiling for 5 min. The cell lysates were separated by polyacrylamide gel electrophoresis using 8–16% Tris/glycine polyacrylamide gradient gels (Novex, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA), and the membranes were subjected to immunoblotting with phospho-specific antibodies that recognize the phosphorylated and thereby activated forms of p38 and ERK MAPKs, respectively (New England Biolabs, Beverly, MA). Antibodies recognizing the active and inactive forms of these MAPKs were used to control that equal amounts of protein were present in all samples. The appropriate antibodies were obtained from Santa Cruz Biotechnology. Secondary horseradish antibody conjugate and the enhanced chemiluminescence assay kit were from Pierce (Rockford, IL).

RESULTS

HS causes ATP release and conversion to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and adenosine

HS-induced cell shrinkage mechanically deforms the plasma membrane, and in a number of cell types, mechanical stress has been shown to release ATP into the extracellular space, where it is converted to ADP, AMP, and adenosine [15–19]. We tested if HS can cause ATP release in PMN. We exposed PMN to different concentrations of HS by adding appropriate amounts of a 1M NaCl solution in HBSS and then assayed extracellular concentrations of adenine nucleotides by HPLC analysis. We found that HS caused a dose-dependent release of ATP from PMN, and control cells in the absence of HS released only minimal amounts of ATP (**Fig. 1A**). Addition of 100 mM HS increased extracellular ATP and ADP concentrations to seven and five times the respective values of unstimulated cells. ATP and ADP reached maximum levels of 17.4 and 2.4 nmol/2.5 × 10^6 cells, respectively, within 5 min after HS stimulation. The concentrations of AMP and adenosine increased 15- and fourfold, reaching 27.6 and 3.6 nmol/2.5 × 10^6 cells, respectively, over a period of 15 min after exposure to HS (Fig. 1B). These patterns are consistent with a precursor-product relation indicative of ATP conversion by ecto-ATPases and related enzymes that have been observed for a variety of cells [12, 19, 20].

ATP and its products differentially regulate PMN function

HS can block O_2^- formation of fMLP-stimulated PMN by inhibiting several signaling pathways downstream of the fMLP receptor in a manner that depends on cAMP/PKA [8, 9]. ATP and its degradative products, including adenosine, can engage P2 and P1 receptors that are members of the G protein-coupled receptor family and could be responsible for triggering cAMP accumulation and PKA activation in PMN. Therefore, we investigated the effect of ATP and its products on fMLP-promoted O_2^- formation (**Fig. 2**). PMN were exposed for 10 min to increasing concentrations of ATP, ADP, AMP, adenosine, or HS and stimulated with 100 nM fMLP, and O_2^- formation was determined using a cytochrome C reduction assay. We found that 1–10 nM ATP suppressed fMLP-induced O_2^- formation by 20%, and higher ATP concentrations (1–1000 μM) augmented O_2^- formation by up to 300% (Fig. 2A). The enhancing effect of ATP was obliterated by the addition of apyrase (20 U/ml), an enzyme that cleaves adenine nucleotides. HS suppressed PMN function by 100% at a hypertonicity level of 100 mM with an IC_{50} value of 20 mM (Fig. 2B). In contrast to the results with ATP, AMP and adenosine suppressed fMLP-induced O_2^- formation by 70–80%, and IC_{50} values were 2 μM and 10 nM,

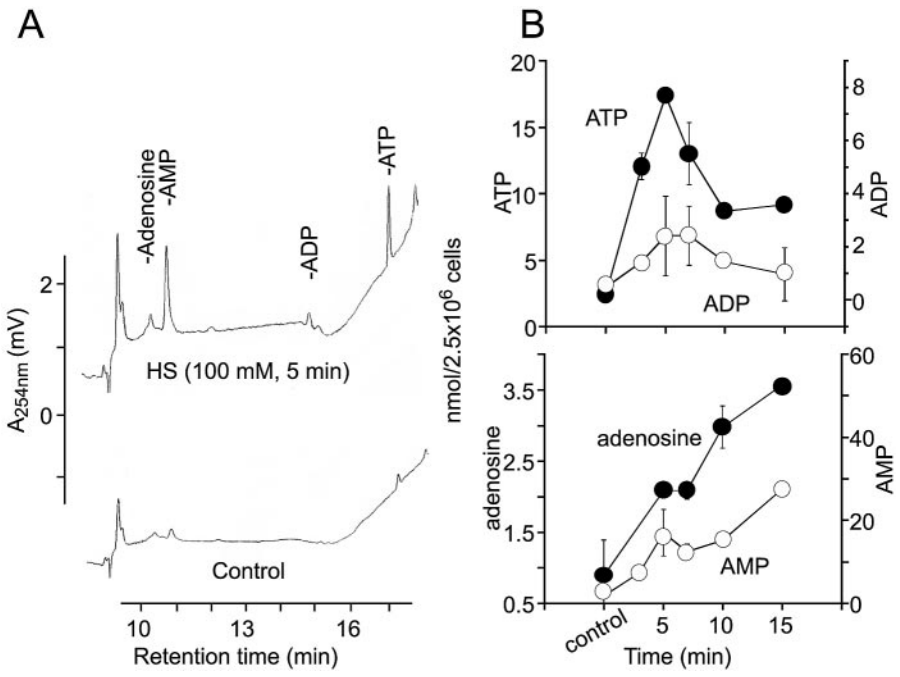

Fig. 1. HS causes ATP release and conversion to ADP, AMP, and adenosine. PMN (250 μl at 10^7/ml) were exposed to HS, PMN suspensions were sampled at indicated time-points, and ATP, ADP, AMP, and adenosine were measured in the supernatants with HPLC. (A) Representative chromatograms of supernatants obtained 5 min after addition of hypertonic (100 mM, upper trace) or isotonic HBSS (lower trace). A detailed description of the separation method can be found in Materials and Methods. (B) Extracellular concentrations of ATP, ADP (upper panel), AMP, and adenosine (lower panel) at different time-points after stimulation of neutrophils with 100 mM HS. Data shown are representative of three individual experiments with cells from different donors.

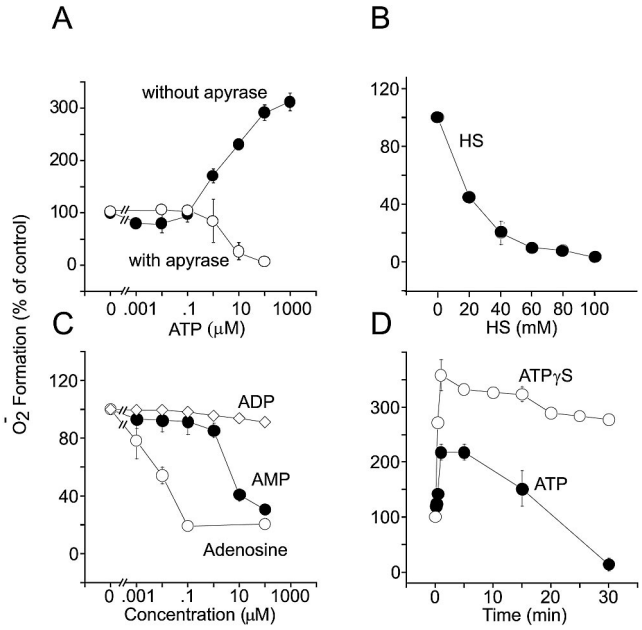

Fig. 2. ATP and its products differentially regulate PMN function. (A) Neutrophils (2×10^6/ml) were treated with (○) or without (●) apyrase (20 U/ml) for 30 min. The cells were then exposed to increasing concentrations of ATP for 10 min and stimulated with 100 nM fMLP, and superoxide formation was measured with a cytochrome C reduction assay. (B and C) Cells were stimulated with increasing concentrations of HS, ADP, AMP, or adenosine, and fMLP-induced superoxide formation was determined as above. (D) Cells were incubated for increasing periods of time with 100 μM ATP or the nonhydrolyzable form, ATPγS, and fMLP-induced superoxide formation was determined as above. O_2^- formation is expressed as the percentage of the maximum response to 100 nM fMLP of control cells under isotonic conditions. Values are expressed as mean ± SD of experiments performed in duplicate; the data shown are representative of three individual experiments with cells from different donors.

respectively (Fig. 2C). These findings show that ATP enhances PMN function, and the ATP metabolites have opposite actions, implying that the kinetics of ATP release and its catabolism by ATPases may account for the differential regulation of PMN functions by hypertonic stress. As low concentrations of ATP suppressed PMN function, but higher concentrations did not, we hypothesized that ATP released by HS is rapidly converted to adenosine, thereby suppressing PMN function. As one test of this hypothesis, we compared the response of PMN to ATP and the nonhydrolyzable ATP analog ATPγS. Exposure to 100 μM ATP or ATPγS rapidly increased fMLP-induced O_2^- formation, but although the stimulation by ATP converted to a suppression over an incubation period of >15 min, the stimulation by ATPγS diminished only slightly (Fig. 2D). These results are consistent with the idea that extracellular ATP enhances, and metabolism of this ATP to adenosine suppresses PMN function. In Figure 2D, the conversion of the 100 μM ATP to effective concentrations of its suppressive products seemed to require ~20 min of expose to PMN. However, significant levels of suppressive ATP products appear to be produced within 10 min of exposure to HS (Fig. 2B). The apparent paradox between the time requirements in Figure 2, B and D, suggests that the local concentrations of ATP, the dynamics of ATP release, and the diffusion of ATP and its products between the cell surface and the bulk media contribute to the functional response of PMN to ATP.

Adenosine is required for suppression of PMN by HS

Based on the observations above and as responses to AMP and adenosine closely mimicked the effect of HS, we tested the hypothesis that HS-induced PMN suppression involves ATP release and the subsequent conversion of released ATP to AMP and adenosine. To test the role of adenosine in the suppression of PMN by HS, we treated PMN with increasing concentrations of HS in the presence or absence of adenosine deaminase (ADA; 3 U/ml) to scavenge extracellular adenosine. PMN were then stimulated with fMLP, and superoxide formation was measured. ADA completely abolished the suppressive effects of HS (**Fig. 3**), a result consistent with the idea that HS-promoted release of ATP and its conversion to adenosine are required for the suppression of PMN function by HS. Thus, HS suppresses PMN superoxide formation and possibly other properties of PMN, including motility, chemotaxis, and phagocytosis through the action of adenosine [12, 21–23].

HS suppresses PMN through activation of A2 adenosine receptors

To obtain further information about the role of adenosine in the osmotic suppression of PMN, we assessed adenosine receptor expression in human PMN. Immunofluorescence microscopy with antireceptor antibodies showed that all four known adenosine receptors (A1, A2a, A2b, and A3) and P2X7 and P2Y2 receptors are expressed on the surface of PMN (**Fig. 4**).

Using real-time RT-PCR, we detected mRNA encoding all four adenosine receptors (**Fig. 5A**). The relative abundance of mRNA copies encoding the A1 and A3 receptors was highest, followed by that of the A2a and the A2b receptors. We used selective agonists and antagonists of the adenosine receptor subtypes to define their contributions to the regulation of fMLP-induced O_2^- formation by HS and adenosine. Neutrophils were stimulated for 5 min with increasing concentrations of the A1, A2, and A3 receptor-specific agonists CPA, CGS-21680, and IB-MECA, respectively. PMN were then activated with 100 nM fMLP, and superoxide formation was measured.

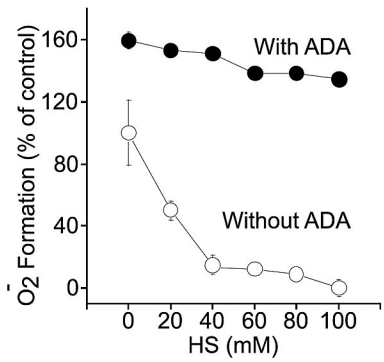

Fig. 3. Cells were treated with increasing concentrations of HS in the presence (●) or absence (○) of 3 U/ml ADA to scavenge extracellular adenosine, and fMLP-induced superoxide formation was assessed as above. O_2^- formation is expressed as the percentage of the maximum response to 100 nM fMLP of control cells under isotonic conditions. Values are expressed as mean ± SD of experiments performed in duplicate; the data shown are representative of three individual experiments with cells from different donors.

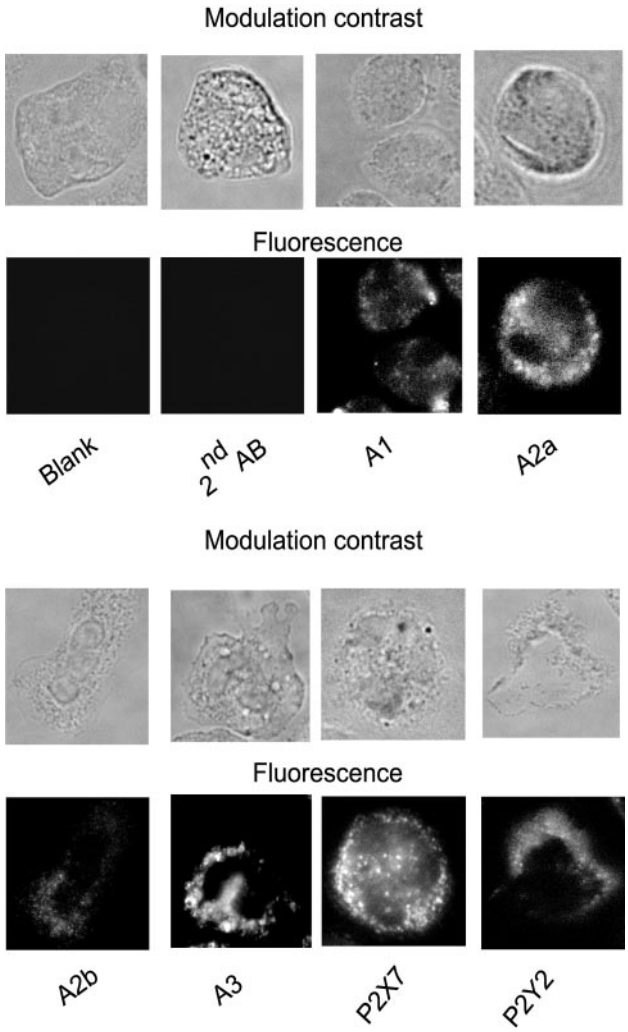

Fig. 4. Adenosine and P2 receptor expression on human PMN. Isolated human neutrophils were fixed, permeabilized, and stained with antibodies to the A1, A2a, A2b, A3, P2X7, and P2Y2 receptors followed by fluorescein isothiocyanate (FITC)-labeled secondary antibodies as described in Materials and Methods. Control cells were stained without any antibody (Blank) or without irrelevant primary antibody followed by the FITC-labeled antibody (2^{nd} AB). Fluorescence (lower) and modulation contrast (upper) image pairs were acquired at 100× original magnification.

The A2 agonist CGS-21680 inhibited O_2^- formation by >97% at a concentration of 10 μM (IC_{50} value of 1 nM), and the A1 and A3 receptor agonists CPA and IB-MECA (Fig. 5B) were inactive. Thus, in spite of the higher expression of A1 and A3 receptor mRNA, A2 receptor activation seems to be a key mechanism required for the suppression of PMN function by HS or adenosine.

We tested this concept further with adenosine receptor antagonists. PMN were exposed for 30 min to increasing concentration of adenosine receptor-selective antagonists, exposed to 20 mM HS for 10 min, and stimulated with 100 nM fMLP, and then superoxide formation was determined. These experiments confirmed the key role of A2 receptors in the suppression of O_2^- formation by HS (Fig. 5C). The A1 and A3 antagonists MRS-1191 and DPCPX had little effects on the suppression of superoxide formation by HS. In contrast, the general adenosine receptor antagonist 8-SPT and the specific A2 antagonists DMPX and the A2a- and A2b-specific antagonists CSC and enprofylline reversed the suppressive effect of HS. These findings suggest that both A2 receptors contribute to the suppression of PMN by HS.

HS suppresses PMN by cAMP accumulation through A2a and A2b adenosine receptors

We have previously shown that HS rapidly increases intracellular cAMP levels by up to sevenfold [10]. The concentration of cAMP correlated with the applied level of hypertonicity and the degree of suppression of PMN function. Based on these findings and the results shown above, we hypothesized that HS causes cAMP accumulation through the activation of A2a and A2b receptors, as both are G_s-coupled receptors. To explore this possibility, we tested if A2 receptor antagonists could prevent the activation of cAMP by HS. We pretreated PMN with the A2a receptor antagonist CSC (1 μM) or the A2b receptor antagonist enprofylline (10 nM) for 30 min, stimulated the cells with 20 mM HS in the presence of 80 μM of the phosphodiesterase inhibitor IBMX for 10 min, and measured cAMP concentration. Unstimulated cells and cells that were

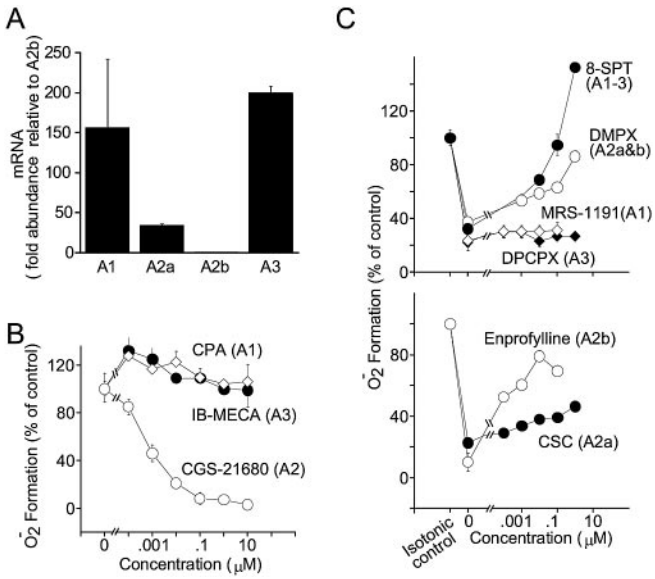

Fig. 5. HS suppresses PMN through A2 adenosine receptor activation. (A) The relative abundance of mRNA copies of the different receptor subtypes in human PMN was assessed by real-time RT-PCR analysis using the value for A2b for normalization. PMN possess mRNA transcripts of all four known P1 receptors A1, A2a, A2b, and A3. (B) Neutrophils were pretreated for 5 min with increasing concentrations of the A1 adenosine receptor agonist CPA, the A2 receptor agonist CGS, or the A3 receptor agonist IB-MECA. Then the cells were exposed to 100 nM fMLP, and superoxide formation was measured. A2 receptor agonist, but not the A1 and A3 receptor agonists, mimicked the effect of HS. (C and D) Neutrophils were pretreated for 30 min with increasing concentrations of the indicated adenosine receptor antagonist with different specificities toward the various receptor subclasses (shown in parentheses); then, cells were exposed to 20 mM HS for 10 min and stimulated with 100 nM fMLP, and superoxide formation was measured as described above. Only antagonists with specificities toward A2 receptors were able to reverse the suppressive effect of HS on superoxide formation. In all experiments, superoxide formation is expressed as percentage of untreated control cells that were stimulated with 100 nM fMLP in the absence of HS. Values are expressed as mean ± SD of duplicates, and the data shown are representative of three individual experiments with cells from different donors.

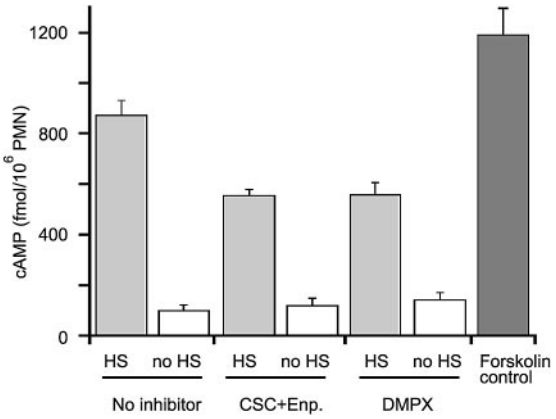

Fig. 6. A2 receptor antagonists reduce HS-induced cAMP accumulation. PMN were pretreated with a combination of the A2a and A2b receptor-specific antagonists CSC (1 μM) and enprofylline (Enp.; 10 nM) or with the A2 receptor-specific antagonist DMPX (1 μM) for 30 min and were exposed to 100 mM HS, and cAMP accumulation was measured. Forskolin-stimulated cells (50 μM) served as positive control. Values are expressed as mean ± SD of triplicate determinations, and the data shown are representative of two experiments with cells from different donors.

stimulated with the cAMP-elevating drug forskolin served as negative and positive controls, respectively. HS (20 mM) augmented cAMP accumulation to levels similar to those we have previously reported [9]. These cAMP levels were significantly reduced by the A2a and A2b receptor antagonists CSC and enprofylline ($P<0.005$) and by the A2-specific antagonist DMPX ($P<0.005$; **Fig. 6**). These data lead us to conclude that HS exerts its suppressive effect on PMN function at least in part by the accumulation of intracellular cAMP through the activation of the two A2 receptors. However, it is possible that other G_s protein-coupled receptors might be involved in the generation of cAMP. For example, very recently, a new P2Y receptor member (P2Y15) has been identified, which binds to adenosine and cAMP and results in the elevation of intracellular cAMP [24].

HS augments PMN function by ATP-induced P2 receptor engagement and MAPK activation

There are ~15 nucleotide receptors known to date, and these are classified as members of the G protein-coupled P2Y or ion-channel P2X groups [14–16]. As HS can enhance PMN function under certain circumstances and as ATP can significantly increase fMLP-induced O_2^- formation (Fig. 2A), we investigated the expression of P2 receptors in PMN. Real-time RT-PCR analysis revealed that human PMN express high levels of mRNA copies that encode the following P2 receptor subtypes (in order of relative abundance): P2Y2, P2Y4, and P2X7 and to a lesser extent, P2Y11 and P2Y6 (**Fig. 7A**). We verified expression of P2X7 and P2Y2 receptors on PMN using immunofluorescence (Fig. 4); antibodies for the other receptors

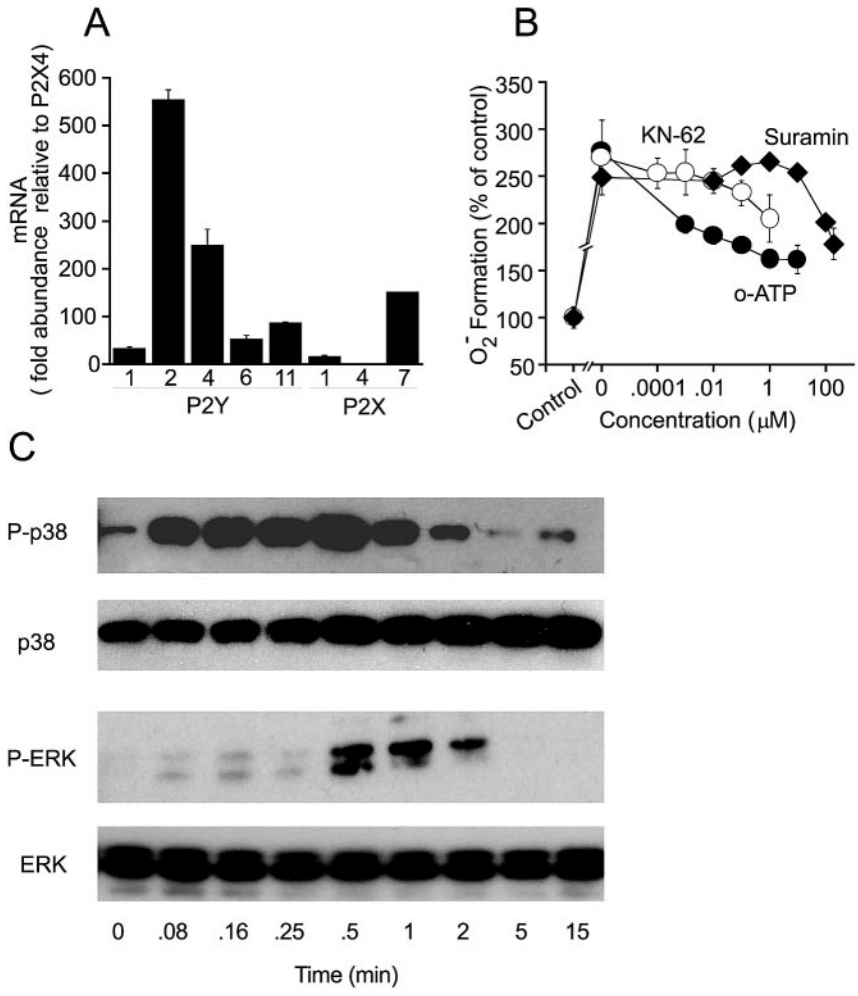

Fig. 7. ATP increases PMN function by activation of P2 receptors. (A) Real-time RT-PCR analysis of the indicated P2 receptor candidates was done as described in Figure 4. Although mRNA transcripts of all receptors were detected, their relative abundance differed greatly, and that of the P2X4 receptor was used for normalization. (B) PMN were treated with the P2 receptor antagonists o-ATP, KN-62, and suramin for 30 min. Then the cells were stimulated with 100 nM fMLP in the presence of 100 μM ATP, and superoxide formation was measured. Data are expressed as percentage of the response of control cells that was stimulated with fMLP in the absence of ATP, and values are expressed as mean ± SD of duplicates. The data shown are representative of three individual experiments with cells from different donors. (C) PMN were stimulated with 100 μM ATP for the indicated periods. Then the cells were lysed, and activation of p38 and ERK MAPKs was determined by immunoblotting the phosphorylated (and thereby activated) forms (P-p38 and P-ERK) of these MAPKs. Total amounts of these MAPKs were assessed to control that equal amounts of the proteins were present in all samples. The data are representative of at least two experiments with cells from different donors.

identified by PCR were not available. To test the role of P2 receptors in the enhancement of oxidative burst by ATP, we treated PMN with increasing concentrations of the P2 antagonists, suramin, o-ATP, and KN-62 for 30 min. Next we added 100 μM ATP for 10 min, followed by stimulation with 100 nM fMLP, and then we measured superoxide formation (Fig. 7B). All three P2 antagonists inhibited the enhancement of fMLP-stimulated O_2^- formation by ATP, suggesting that activation of P2 receptors is responsible for the enhancing effects of HS on PMN function. At concentrations that reversed the ATP response, these P2 antagonists had no discernible effect on superoxide formation of cells that was stimulated with fMLP in the absence of exogenous ATP (data not shown).

The activation of ERK and p38 MAPKs is a key event in the stimulation of neutrophil responses, and our previous work has shown that HS preferentially activates p38 MAPK [8]. Therefore, we tested whether extracellular ATP activates these MAPKs in PMN. Neutrophils were exposed to 100 μM ATP, and activation of the ERK and p38 MAPKs was assessed by immunoblotting with antibodies that recognize the phosphorylated (activated) forms of these kinases. Incubation with ATP dose-dependently activated both MAPKs (data not shown); activation of p38 MAPK was particularly rapid with maximum response between 5 and 30 s (Fig. 7C). As both MAPK family members are activated by fMLP, and p38 MAPK activation is involved in the enhancing effects of HS [8], these findings suggest that ATP serves as a costimulatory intermediate. ATP may enhance chemoattractant-stimulated neutrophil responses through P2 receptor activation, which in turn reinforces the stimulatory signals that are transmitted via MAPK pathways.

DISCUSSION

In recent years, the use of hypertonic fluids as a therapeutic approach to regulate immune and inflammatory cell functions, particularly after trauma, has attracted considerable interest [2, 4]. Experimental and clinical evidence suggests that such fluids show promise as a means to decrease lymphocyte suppression and anergy after trauma and reduce neutrophil activation and host tissue injury in inflammatory diseases [2–8]. We have previously shown that hypertonic stress regulates PMN functions regardless of whether NaCl, KCl, sucrose, or choline chloride is used to increase the osmolarity of the extracellular medium [8].

A number of studies have focused on the signaling mechanisms through which osmotic stress regulates the functions of immune cells and other mammalian cells. However, the nature of the receptors that mediate recognition and response of these cells to osmotic stress has remained poorly defined. Here, we show that physiologically relevant osmotic stress causes the regulated release of ATP from PMN and that ATP and its products can increase or suppress neutrophil responses through autocrine-feedback regulation via P2 and P1 receptors. The findings complement and extend previous work that has evaluated the impact of ATP and adenosine on PMN responses [20–23, 25]. It is interesting that it has been shown that the A2a adenosine receptor has a nonredundant role in the attenuation of inflammation and tissue damage in a mouse model of septic shock [26]. Together with these data, our findings offer an intriguing model to explain how HS can enhance and suppress PMN functions (**Fig. 8**). In this model, hypertonic stress shrinks PMN and causes the release of intracellular ATP. Adenosine, derived by conversion of the released ATP, activates A2 receptors that stimulate adenylyl cyclase activity and increase intracellular cAMP accumulation; the increase in cAMP then blocks PMN activation, presumably via PKA and PKA-promoted phosphorylation of key target proteins. Under conditions where extracellular ATP concentration is favored relative to that of adenosine, PMN functions are enhanced by P2 receptor activation and in turn, the activation of p38 and ERK MAPKs.

This type of osmoreceptor system may be widely expressed, as other cell types, including endothelial and epithelial cells, smooth muscle cells, fibroblasts, hepatocytes, and T cells, release ATP in response to mechanical or osmotic stress [15, 17, 18]. In the case of T cells, we have previously shown that HS-induced ATP release increases interleukin-2 expression through the activation of P2X receptors [15]. Thus, T cells appear to possess an osmoreceptor system that is similar to the one described here for PMN. It remains to be seen, however, whether the conversion of ATP to adenosine plays a role in the response of T cells to osmotic stress.

The precise nature of the adenosine and P2 receptors that are involved in mediating the inhibitory and stimulatory responses observed in the response of PMN to HS will require further study. It is intriguing that PMN appear to express a large number of receptors capable of recognizing adenosine and ATP as well as other nucleotides. In spite of the higher

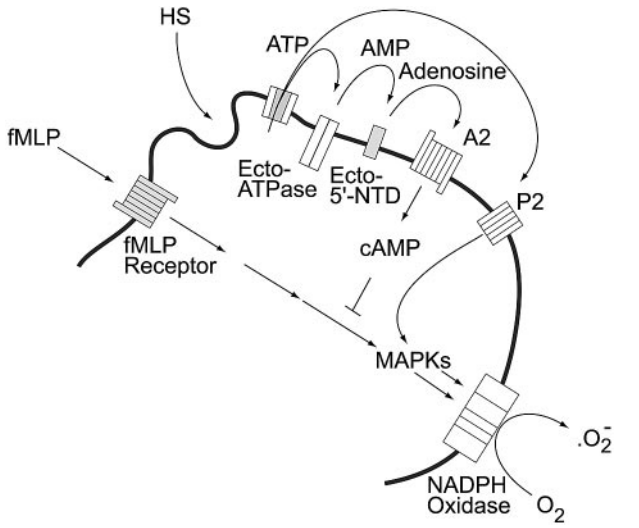

Fig. 8. Proposed model of the mechanisms through which HS regulates PMN functions. The results of our study suggest an osmoreceptor system through which HS regulates PMN function via the release of ATP. Released ATP can engage P2 receptors that costimulate PMN function by ERK and p38 MAPK activation. However, external ATP is also quickly converted to adenosine, which engages A2 receptors that generate cAMP and blunt PMN function. These opposing processes are balanced by the mechanisms that control ATP release, the enzymes that convert ATP to adenosine, and the availability of P2 and A2 receptors, and all these parameters define whether HS augments or suppresses PMN functions. Ecto-5′-NTD, Ectoenzyme 5′-nucleotidase; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

expression of A1 and A3 mRNA, studies with antagonists suggest that A2 receptors are responsible for the suppression of PMN functions by HS. Based on abundance of mRNA and known properties for recognition of nucleotides, the stimulatory P2 receptors of PMN that respond to ATP might be P2Y2, P2X7, or perhaps P2Y11. These receptors, in particular P2X7, are known to be inhibited by the three antagonists (suramin, o-ATP, and KN-62) used in the current study and have been implicated in other immunostimulatory and proinflammatory activities [15, 27, 28].

We found relatively low concentrations of extracellular ATP with a maximum of ~17 nmol/2.5 × 10^6 cells released in response to osmotic stimulation of PMN (Fig. 1B). This value is lower than the value reported by Eltzschig et al. [29], who detected a maximum level of 215 nmol ATP released by 10^7 PMN in response to fMLP stimulation. These differences may be a result of the different agents used to evoke ATP release from PMN or differences in the preparation and handling of cells. According to the results shown in Figure 2A, the concentration of ATP that we and Eltzschig et al. [29] detect would modestly suppress superoxide formation. However, in addition to ATP, the cell supernatants of HS-treated cells would also contain concentrations of AMP and adenosine, which would further suppress PMN function. In addition, the concentrations of these molecules are likely to be highest in the vicinity of the cell membrane, where ATP is released and converted, and concentrations assessed in the bulk phase, as we have done here with HPLC, are considerably lower, as previously shown in other cell systems [30]. The inverse is likely to be true for the experiments wherein we studied the effects of exogenous ATP, AMP, and adenosine on PMN responses, where the concentrations of these substances are likely higher in the supernatant than close to the cell membrane.

The model proposed in Figure 8 suggests an important role for the enzymes that catalyze the conversion of ATP to ADP, AMP, and adenosine. The relative activities and localization of these enzymes, as well as those of the P1 and P2 receptors, in relation to the sites where ATP is released represent critical parameters that may determine whether osmotic stress stimulates or suppresses PMN functions. In addition, the availability and distribution of these molecules may determine how PMN respond to ATP from other sources. Damaged and dying cells and microbes also release ATP, which may increase the recruitment of scavenging PMN to inflammatory or infectious sites and regulate PMN function through ATP and adenosine receptor activation. It is interesting that the ancient method of applying honey, sugar, salt, or other hyperosmolar agents to infected wounds could have its basis in the regulation of such defense mechanisms through the osmoreceptor system we identified in neutrophils, perhaps by augmenting neutrophil responses to the chemical signals obtained from invading microbes.

Several recent studies have shown that A2 receptor activation can be used to control inflammation in vivo [22, 23, 26]. It is intriguing to speculate that anti-inflammatory actions of hypertonic fluids could be based on the same principle of A2 receptor activation. Additional studies are required to define the exact P1 and P2 receptor types and the mechanism of their differential activation by osmotic stress. This knowledge may make it possible to therapeutically fine-tune neutrophil responses so as to increase or prevent cell activation and thereby assist in the treatment of infectious and inflammatory diseases.

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