Hypertonic saline enhances neutrophil elastase release through activation of P2 and A3 receptors

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Hypertonic saline enhances neutrophil elastase release through activation of P2 and A3 receptors. Am J Physiol Cell Physiol 290: C1051–C1059, 2006. First published November 9, 2005; doi:10.1152/ajpcell.00216.2005.—Hypertonic saline (HS) holds promise as a novel resuscitation fluid for the treatment of trauma patients because HS inhibits polymorphonuclear neutrophil (PMN) activation and thereby prevents host tissue damage and associated posttraumatic complications. However, depending on conditions of cell activation, HS can increase PMN degranulation, which could exacerbate tissue damage in trauma victims. The cellular mechanism by which HS increases degranulation is unknown. In the present study, we tested whether HS-induced ATP release from PMN and feedback via P1 and/or P2 receptors may be involved in the enhancement of degranulation by HS. We found that HS enhances elastase release and ERK and p38 MAPK activation when HS is added after activation of PMN with formyl peptide (fMLP) or phorbol ester (PMA). Agonists of P2 nucleotide and A3 adenosine receptors mimicked these enhancing effects of HS, whereas antagonists of A3 receptors or removal of extracellular ATP with apyrase diminished the response to HS. A1 adenosine receptor antagonists increased the enhancing effect of HS, whereas A1 receptor agonists inhibited elastase release. These data suggest that HS upregulates degranulation via ATP release and positive feedback through P2 and A3 receptors. We propose that these feedback mechanisms can serve as potential pharmacological targets to fine-tune the clinical effectiveness of HS resuscitation.

resuscitation; inflammation; osmotic stimulation; nucleotide receptor signaling

POLYMORPHONUCLEAR NEUTROPHILS (PMN) play a crucial role in the defense against invading bacteria, fungi, and protozoa. The defensive arsenal of PMN includes a large number of proteolytic enzymes stored in intracellular granules that can be released through a process referred to as degranulation or exocytosis (38). These enzymes are needed to attack and digest target microorganisms. However, the same enzymes can cause severe collateral damage to tissues of host organs in the course of inflammatory diseases. In trauma patients, PMN activation in response to ischemia and reperfusion often leads to tissue damage of the lungs and to severe posttraumatic complications such as adult respiratory distress syndrome (ARDS) and multiple organ failure syndrome (24, 39).

Elastase stored in the azurophil granules of PMN is a notorious member of the family of proteolytic enzymes that act as mediators of tissue destruction. Elastase is able to hydrolyze matrix proteins and connective tissue components such as elastin, proteoglycan, and fibronectin (38). Thus the release of elastase in the absence of naturally occurring functional protease inhibitors is responsible for tissue destruction under pathological conditions such as emphysema, ARDS, hemorrhage/reperfusion injury, and septic shock (11, 17, 34, 39).

Since its introduction in the beginning of the last century, fluid resuscitation for the treatment of hemorrhagic shock has been carried out with normal saline or Ringer solution. These fluids were designed to approximate the tonicity of human plasma. In recent years, interest in the concept of small-volume resuscitation with hypertonic saline (HS) has grown because hypertonic fluids can be easily transported and infused and because they restore blood pressure more rapidly than isotonic fluids due to shifts of intracellular and extravascular fluid into the vasculature. Hypertonic fluid resuscitation involves the infusion typically of 4 ml/kg body wt of a 7.5% NaCl solution that can contain colloids such as dextran (29, 36). Animal models and experiments with isolated human PMN have shown that HS can reduce the risk of posttraumatic complication by suppressing excessive PMN activation (1, 14, 26).

We have studied the mechanisms by which HS suppresses PMN activation and found that HS induces a rapid release of cellular ATP. Released ATP is then degraded to adenosine, which activates A2 adenosine receptors that block fMLP receptor signals through cAMP-mediated pathways (5, 14, 22). Interestingly, our group and others also have found that HS not only can suppress PMN functions but also can enhance degranulation depending on the timing of HS addition relative to PMN activation with stimuli such as formyl peptide (fMLP) (14, 23). The mechanisms by which HS enhances PMN function are unknown. In the present study, we hypothesized that HS-induced ATP release could be involved in the enhancement of PMN degranulation through positive feedback mechanisms that involve P1 adenosine and/or P2 nucleotide receptors.

MATERIALS AND METHODS

Materials. 8-(p-Sulfophenyl)theophylline (8-SPT), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 3,7-dimethyl-1-(2-propynyl)xanthine (DMPX), 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-phenyl-1,4-(38)-dihydropyridine-3,5-dicarboxylate (MRS-1191), N°-cyclopentyladenosine (CPA), 2-p-(2-carboxyethyl)phenethylaminio-5°-N-ethylcarboxamidoadenosine hydrobromide-hemicarbonate salt (CGS-21680), N°-(3-isodobenzyl)adenosine-5°-N-methyluronamid (IB-MECA), and cytochalasin B were obtained from Sigma-Aldrich (St. Louis, MO). Periodate-oxidized ATP (o-ATP), 1-[N,O-bis(5-isouquinolinesulfonfyl)-N-methyl-l-tyrosyl]-4-phenylpirazone (KN-62), pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), ATP, adenosine 

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S’-O-(3-thiotriphosphate) (ATPγS), and suramin were obtained from Calbiochem (San Diego, CA). Dextran 500 and Percoll were obtained from Pharmacia (Piscataway, NJ).

Isolation of human PMN. The Human Research Program of the University of California, San Diego, approved all experiments described in this study. Peripheral venous blood was obtained from healthy human volunteers. PMN were isolated from peripheral blood as described previously (14). Briefly, heparinized blood was mixed with 5% (wt/vol) Dextran 500 dissolved in a normal saline solution and allowed to sedimentate for 30 min at room temperature. The cells in the supernatant were separated with Percoll gradient centrifugation according to the manufacturer’s recommendations. The PMN layer was collected and washed twice with Hanks’ balanced salt solution (HBSS; Irvine Scientific, Santa Ana, CA). PMN were suspended in HBSS at a concentration of 1 × 10⁶ PMN/ml and used for experiments immediately. Cell isolation and all subsequent experiments were performed under sterile and pyrogen-free conditions.

Hypertonic stimulation. Culture media were made hypertonic by adding appropriate volumes of hypertonic HBSS that contained an additional 1 M NaCl, raising its total Na⁺ concentration from 154 to 1,154 mM. For example, in experiments indicating a hypertonicity of 40 mM, we raised the extracellular Na⁺ concentration from 154 mM to a total of 194 mM by adding 40 μl culture medium of the hypertonic HBSS described above.

Elastase release. PMN (1 × 10⁶) were preincubated for 10 min at 37°C with 5 μl cytochalasin B as described previously (21). The cells were then stimulated with 100 nM fMLP or treated as described in the experiments below. After stimulation for 1 h, cells were placed on ice for 10 min and centrifuged at 16,000 g for 10 s. Elastase activity released into the supernatant was measured as described previously (28). Briefly, 40 μl of supernatant was mixed with 160 μl of a buffer consisting of 50 mM Tris-HCl and 100 mM NaCl, pH 7.4, containing 0.05% (vol/vol) Triton X-100. Enzymatic reactions were started by the addition of the elastase-specific chromogenic substrate N-methoxy-2-naphthyl acetate (Sigma) at a final concentration of 1 mM. After 30 min at room temperature, the change in optical density was measured at a wavelength of 405 nm. Cytochalasin B was used to increase elastase release in response to fMLP. Under isotonic conditions, in the presence of cytochalasin B, stimulation with 100 nM fMLP or 1 ng/ml PMA caused the release of 48.6 ± 5.9 or 7.4 ± 5.0%, respectively, of the total amount of elastase activity contained within the cells. In the absence of cytochalasin B, cells stimulated with fMLP or PMA only released 3.75 ± 0.68 or 1.28 ± 0.36%, respectively, under these conditions. Cytochalasin B treatment did not affect the ability of cells to respond to osmotic stress with cell shrinkage and ATP release.

High-performance liquid chromatography. The kinetic properties of hydrolysis of exogenous ATP by apyrase were studied with high-performance liquid chromatography (HPLC). Apyrase and ATP were incubated at 37°C for different periods, and changes of the concentrations of ATP and of its hydrolysis products were assessed using HPLC analysis. Samples were then boiled for 10 min to stop enzymatic reactions and centrifuged at 4°C and 16,000 g for 1 min, and concentrations of ATP, ADP, AMP, adenosine, and inosine were analyzed using HPLC as previously described (5).

Measurement of MAPK activation. To measure p38 and ERK MAPK activation, we placed purified PMN (1 × 10⁶), stimulated as described below, into an ice bath for 10 min to stop all enzymatic reactions. Typically, samples reached a temperature of 4°C within 3 min. This delay should be taken into account when comparing our data with the findings of researchers who use other, more rapid methods to stop reactions in their preparations of cell lysates for immunoblotting. Cells were centrifuged at 10 s at 16,000 g and 4°C, and the cell pellets were boiled for 5 min in 100 μl of Tris-glycine-SDS sample buffer (Invitrogen, Carlsbad, CA) containing 100 mM dithiothreitol. Each sample (12 μl) was then separated by SDS-PAGE using a 1-mm-thick 12% Tris-glycine-polyacrylamide gel (Novex, San Diego, CA). Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, 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 MAPK, respectively (Cell Signaling Technology, Beverly, MA). The secondary horseradish-conjugated antibody and the enhanced chemiluminescence assay kit were obtained from Pierce (Rockford, IL). Membranes were stripped and reprobed with antibodies that recognize the active as well as inactive forms of p38 and ERK MAPK. Using this method and total protein staining, we were able to verify that equal amounts of protein were present in all samples depicted throughout this manuscript.

RESULTS

HS has suppressive and enhancing effects on PMN degranulation. Freshly isolated PMN were exposed to increasing concentrations of HS 5 min before or after stimulation with fMLP or 5 min after stimulation with PMA, and then elastase release was determined (Fig. 1). HS in the absence of additional stimuli had no significant effect on elastase release. When HS was added to PMN before stimulation with fMLP, the fMLP-stimulated elastase release was inhibited in a dose-dependent manner (Fig. 1A). HS concentrations >20 mM inhibited elastase release by >50%. In contrast, HS added after fMLP stimulation of PMN enhanced elastase release in a dose-dependent manner with an EC₅₀ value of ~40 mM HS (Fig. 1A). Although HS pretreatment inhibited elastase release in response to fMLP, it was unable to prevent PMA-induced PMN responses (14) and actually enhanced elastase release of PMN stimulated with PMA (Fig. 1B). This finding indicates that HS-induced suppression depends on an interference with activation signaling upstream of PKC, a class of signaling proteins directly activated by PMA. In summary, our data show that HS can exert two opposing effects on PMN degranulation: 1) a suppressive effect that requires pretreatment of PMN with HS before stimulation with fMLP and that seems to depend on an interference with activation signals upstream of PKC, and 2) an enhancing effect that appears to costimulate elastase release of previously activated PMN.

ERK and p38 MAPK activation parallel effects of HS on degranulation. ERK and p38 MAPK play important roles in the activation of several PMN functions, including degranulation (21, 30, 37). We investigated whether HS could augment the activation of ERK and p38 MAPK in response to fMLP. Increasing concentrations of HS were added to PMN either 2 min before or after stimulation with 100 nM fMLP, and p38 and ERK activation were determined by immunoblotting with phospho-specific antibodies (Fig. 2). HS augmented p38 and ERK activation in a dose-dependent fashion when HS was added to previously stimulated PMN (Fig. 2A). Conversely, HS inhibited p38 and ERK activation when HS was added before cell stimulation with fMLP (Fig. 2B). These data indicate that HS regulates PMN degranulation by influencing signaling mechanisms upstream of ERK and p38 MAPK. In contrast to Tuluc et al. (33), who described maximum p38 MAPK activation at 1 min after cell stimulation, we found that p38 phosphorylation peaked at 0.5 min and that little p38 remained phosphorylated at later stages. These differences in timing of peak p38 activation may be due to discrepancies in the methods used to stop phosphorylation and dephosphorylation reactions.
P2 nucleotide receptor stimulation mimics enhancing effects of HS. In a number of different cell types, mechanical stress induces the release of ATP into the extracellular space (2, 18). We have shown that HS-induced cell shrinkage and mechanical perturbations release ATP from PMN into the extracellular space, where ATP together with its hydrolysis product adenosine modulate cell functions through autocrine feedback loops that involve P2 nucleotide and P1 adenosine receptors (5). To test whether the enhancement of degranulation by HS could involve similar feedback loops, we stimulated PMN with fMLP for 5 min and then added increasing concentrations of ATP or the nonhydrolyzable ATP analog ATPγS to simulate the effect of HS. ATP and ATPγS dose-dependently increased elastase release (Fig. 3). In contrast to ATPγS, however, ATP at concentrations lower than or equal to 1 μM inhibited elastase release, whereas higher ATP concentrations enhanced fMLP-stimulated degranulation (Fig. 3A). Moreover, ATPγS was more effective than ATP in enhancing PMA-stimulated elastase release (Fig. 3B). The observation that low concentrations of ATP suppress PMN degranulation, whereas higher concentrations do not, indicates that ectoenzymes quickly degrade extracellular ATP and that the hydrolytic products of ATP may counterbalance the enhancing effects of ATP. This explanation is consistent with the finding that similar concentrations of the nonhydrolyzable ATP analog ATPγS had stronger enhancing effects than ATP. Thus feedback regulation of PMN degranulation by released ATP appears to depend on several factors: 1) the concentration of extracellular ATP and that of its hydrolysis products, primarily adenosine, 2) the kinetics of ATP hydrolysis, and 3) the surface expression pattern of the P2 and P1 receptors that respond to ATP and its hydrolysis products.

HS enhances PMN degranulation through ATP release. The data demonstrate that extracellular ATP can augment degranulation of previously stimulated PMN (Fig. 3). To test whether HS-induced ATP release is responsible for the upregulation of degranulation by HS, we assessed PMN degranulation in the presence of increasing concentrations of apyrase, an enzyme that catalyzes the breakdown of extracellular ATP. Cells pretreated with apyrase were stimulated with PMA (1 ng/ml) for 5 min and exposed to 40 mM HS (Fig. 4A). Apyrase dose-dependently abolished the enhancing effect of HS, suggesting...
that this enhancing effect depends on the release of ATP into the extracellular space. HPLC analysis of the kinetics of ATP hydrolysis by apyrase revealed rapid conversion of ATP to inosine (Fig. 4B). Interestingly, this reaction did not result in the formation of measurable quantities of ADP, AMP, or adenosine. Together with the findings shown in Fig. 4, this implies an important role for ATP release in the upregulation of degranulation by HS. However, these results do not allow us

Fig. 3. P2 receptor stimulation mimics the enhancing effects of HS on PMN degranulation. A: increasing concentrations of ATP (●) or adenosine 5'-O-(3-thiotriphosphate) (ATPβS; ○) were added to PMN 5 min after stimulation with 100 nM fMLP, and elastase release was determined. B: PMN were stimulated with 1 ng/ml PMA for 5 min followed by 10 μM ATP or ATPβS, and elastase release was measured. Values are expressed as percentages of the maximum response in the absence of ATP or ATPβS and are presented as means (SD) of experiments performed in duplicate. The data shown are representative of 3 individual experiments with cells from different donors.

Fig. 4. HS enhances PMN degranulation by the activation of P2 receptors. A: cells were pretreated with increasing concentrations of apyrase, the cells were stimulated with 1 ng/ml PMA for 5 min before addition of 40 mM HS, and elastase release was determined. B: apyrase (10 U/ml) was incubated with ATP (5 μM) for different time periods, and the concentrations of ATP, ADP, AMP, adenosine (ADO), and inosine were determined using HPLC. C: PMN were pretreated with suramin (100 μM), periodate-oxidized ATP (o-ATP; 10 μM), pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; 10 μM), or KN-62 (1 μM), stimulated with 1 ng/ml PMA for 5 min, and exposed to 40 mM HS, and elastase release was determined. The data shown are representative of 3 individual experiments with cells from different donors. In A and C, values are expressed as percentages of the maximum response to 1 ng/ml PMA of control cells under isotonic conditions in the absence of apyrase or P2 antagonists and are presented as means (SD) of experiments performed in duplicate.
to determine whether ATP itself or its product adenosine is responsible for the upregulation of degranulation by HS.

Extracellular ATP activates members of the P2 family of nucleotide receptors that are divided into the G protein-coupled P2Y and the ligand-gated ion channel P2X subgroups (25). We used different antagonists of P2 receptors to explore which of these P2 receptor classes are involved in the enhancing effect of HS. As shown in Fig. 4C, pretreatment with suramin but not with o-ATP, PPADS, or KN-62 prevented the enhancement of elastase release by HS. These data point toward a role of suramin-sensitive P2 receptors in the enhancement of PMN degranulation by HS. However, suramin is an inhibitor of P2 receptors as well as of ecto-ATPase activity (5, 25). Therefore, the findings shown in Fig. 4C also could point toward hydrolysis products of ATP such as adenosine as important effectors of enhanced degranulation.

A1 and A3 adenosine receptors have opposing effects on PMN degranulation. Figure 3 shows that low concentrations of ATP but not of ATPγS suppressed PMN degranulation, whereas higher concentrations of ATPγS had a stronger enhancing effect than the same concentrations of ATP. Our group (5) has previously reported that PMN quickly hydrolyze ATP released in response to HS, yielding increased extracellular concentrations of adenosine. Adenosine is the natural ligand of the four known mammalian P1 adenosine receptors A1, A2a, A2b, and A3 (25), and human PMN express all four P1 receptors (5). We examined the roles of these P1 receptors in the regulation of PMN degranulation by HS. PMN were stimulated with fMLP or PMA, followed by specific P1 receptor agonists (CPA for A1, CGS-21680 for A2, IB-MECA for A3, and adenosine for all 4 P1 receptors). The appropriate agonist concentrations were determined in preliminary experiments on the basis of published data (5, 8, 10, 15).

Activation of A1 receptors with CPA (1 μM) and of all four adenosine receptors A1–3 with adenosine (10 μM) suppressed degranulation of fMLP- and PMA-stimulated PMN (Fig. 5). Although stimulation of A2 receptors can prevent fMLP-induced PMN responses (5), the A2a agonist CGS-21680 (1 μM) did not alter degranulation of previously activated PMN (Fig. 5, A and B). In contrast, activation of A3 receptors with IB-MECA (0.1 μM) significantly increased PMN degranulation up to sixfold. These findings show that extracellular adenosine can either enhance or suppress degranulation via the A3 or A1 receptors, respectively.

A3 receptors and HS induce p38 MAPK activation. Our group (14) previously reported that HS, at physiologically relevant concentrations of <100 mM, strongly activates p38 but not ERK MAPK. Because A3 receptor activation mimicked the effect of HS on PMN degranulation (Fig. 5, A and B), we tested whether the A3 receptor agonist IB-MECA would be able to upregulate p38 MAPK activation. PMN were stimulated with 100 mM HS or A3 receptor agonist for different incubation times, and ERK and p38 MAPK activation was determined by immunoblotting. HS and IB-MECA rapidly activated p38 MAPK, whereas both stimuli had only weak effects on ERK MAPK (Fig. 5C). This finding supports the
possibility that A3 receptors could be involved in the upregulation of PMN degranulation by HS.

**A1 receptors downregulate enhancement of degranulation by HS.** To explore this possibility in more detail, we studied the role of the four P1 receptors in the upregulation of PMN degranulation by HS. Cells were pretreated for 5 min with specific antagonists of the different adenosine receptors (8-SPT for all 4 adenosine receptors A1–3; DPCPX for A1, DMPX for A2, and MRS-1191 for A3), stimulated with PMA (1 ng/ml) or fMLP (100 nM) for another 5 min, and exposed to 40 mM HS. Antagonist concentrations in these experiments were determined on the basis of published data (5) and optimized in preliminary experiments (data not shown). Inhibition of the A3 receptor with MRS-1191 (1 μM) completely abolished the enhancing effect of HS, indicating that A3 receptor activation plays a main role in the enhancing effects of HS on degranulation (Fig. 6, A and B). In contrast, inhibition of A1 receptors with DPCPX (10 μM) increased the enhancing effects of HS, suggesting that the A1 receptor may exert an inhibitory effect on PMN degranulation that might counterbalance the enhancing effects of HS. The antagonist of the A2 receptors, DMPX (10 μM), did not alter the effect of HS on elastase release, suggesting that A2 receptors are not involved in the upregulation of degranulation by HS (Fig. 6, A and B). These results are consistent with the data in Fig. 5, which show that A3 receptor activation enhances degranulation, whereas A1 receptors appear to inhibit degranulation. Pretreatment with 8-SPT, which inhibits all four P1 receptor subtypes, only partially increased degranulation of PMN in response to HS (Fig. 6, A and B). This finding suggests that P2 receptors or other mechanisms independent of P1 receptors also may have a role in the enhancement of PMN degranulation by HS. Moreover, these results indicate that the balance between A1 and A3 receptors could control the effect of HS on PMN degranulation.

Because we have observed that the effect of HS on degranulation is paralleled by its effect on p38 MAPK activation (Figs. 2 and 5C), we tested how inhibition of P1 receptors with 8-SPT might affect the activation of ERK and p38 MAPK by HS. We found that pretreatment with 8-SPT dose-dependently increased the activation of both ERK and p38 MAPK activation in response to HS (Fig. 6C). This could suggest that P1 receptors may block P2 receptor-induced ERK activation, a notion that is supported by the fact that the nonhydrolyzable ATP analog ATPγS induced p38 and ERK MAPK activation (Fig. 6D). Together, these findings support our hypothesis that HS exposure of PMN may result in the selective activation of p38 MAPK because of the interference of P1 receptor signals with P2 receptor signaling, possibly through the actions of the A1 receptor subtype.

**DISCUSSION**

Hypertonic saline resuscitation is an effective fluid resuscitation regimen for the treatment of patients with trauma and hemorrhagic shock because it quickly restores blood pressure and circulation to increase peripheral tissue perfusion and...
Osmotic enhancement of PMN degranulation

Oxygen consumption (29). The beneficial properties of HS resuscitation have been demonstrated in animal models and clinical trials (29, 35). In addition to its hemodynamic properties, HS also affects many aspects of the cellular immune response to trauma and hemorrhage. Experiments with isolated PMN and animal models of hemorrhagic shock have shown that HS can suppress PMN function (1, 6, 14, 22). Because excessive PMN activation is involved in major posttraumatic complications (24, 39), HS resuscitation has promise to reduce this risk (27).

Unfortunately, the suppressive effect of HS on PMN function depends on the conditions of cell stimulation. Most importantly, the timing of HS treatment in relation to cell activation determines whether HS can suppress or augment PMN responses. We have shown in this report that HS can markedly enhance degranulation of previously activated PMN (Fig. 1). As a result, HS resuscitation may exacerbate tissue injury in trauma patients. To reduce the risk of tissue damage in patients, it is necessary to understand the cellular mechanisms by which HS increases PMN degranulation.

Previous work in our laboratory (5) has revealed that HS causes PMN to release ATP that is rapidly converted to adenosine and that extracellular adenosine is responsible for the suppression of PMN by activating A2 receptors and the cAMP/PKA pathway. In the present study, we examined the role of ATP release and adenosine in the enhancing effects of HS on PMN degranulation. We found that ATP release and activation of P2 and A3 receptors are required for enhanced degranulation and that these receptors seem to act independently, because ATPaseS, which does not generate adenosine, and specific A3 agonists were able to augment degranulation. On the basis of our current knowledge of how HS affects PMN function, we propose the model shown in Fig. 7 to explain how the different P1 and P2 receptors may interact. HS-induced cell shrinkage releases ATP, which can activate P2 receptors.

Ecto-ATPases and 5'-nucleotidases quickly convert ATP to adenosine that can activate A1, A2, and A3 receptors expressed on the cell surface of PMN. A1 receptors suppress degranulation, apparently by interfering with activation signals downstream of PKC, whereas A3 receptors increase degranulation, perhaps by selectively increasing p38 MAPK activation. A2 receptors block fMLP-induced signals upstream of ERK and p38 MAPK. However, A2 receptor signals seem to have no role in the enhancement of degranulation, and they appear to be unable to affect degranulation of previously stimulated PMN.

Together, these data suggest that HS enhances degranulation predominantly via A3 receptor activation. However, our experiments with apyrase and with antagonists of P1 and P2 receptors indicate that P2 receptors also may play an important role. The relative contributions of these independent signals triggered by P2 and A3 receptors in the enhancing effects of HS is likely to depend on multiple factors such as the kinetics of adenosine formation and hydrolysis by ectoenzymes, the surface expression levels of P2 and A3 receptors, and the localization of these receptors at the sites of ATP release and breakdown. To date, a total of 14 different P2 receptor subtypes of P2X and P2Y subgroups have been cloned and characterized (25). Previous work in our laboratory (5) with real-time RT-PCR analysis has revealed that human PMN predominantly express P2Y2, P2Y4, and P2X7, and to a lesser extent P2Y11 and P2Y6, receptors. However, because of the lack of selective antagonist for these P2 receptors, we are not able to determine which of these receptors are involved in the enhancement of PMN degranulation by HS. Based on the four P2 receptor antagonists used in our study and their reported selectivity profiles, our conclusion is that the P2Y2 receptor is the most likely candidate involved in the enhancement of degranulation by HS (13, 16, 20, 25). However, suramin has been reported to inhibit ATPase activity in addition to blocking P2 receptor activation (4). We have confirmed this effect of suramin on ATPase activity in the present study using HPLC analysis, which revealed that suramin indeed inhibited the conversion of the released ATP to adenosine (data not shown). Therefore, the data shown in Fig. 4 may be due to suppressed P2 receptor signaling or to reduced adenosine formation and a lack of A3 receptor activation. Thus definitive answers regarding the relative roles of P2 vs. A3 receptors in the enhancement of degranulation by HS require additional studies, perhaps with RNA interference approaches, for example.

Cell stimulation with fMLP rapidly activates a number of signaling intermediates, including phospholipase C and PKC (3). PMA can directly activate PKC independently of receptor stimulation. We found that the activation of A1 and A3 adenosine receptors had opposite effects on fMLP-induced PMN degranulation: A1 receptors suppressed degranulation, whereas A3 receptors enhanced it (Figs. 5 and 6). We also found that A1 receptor activation decreased degranulation in

Fig. 7. Proposed model of the mechanisms by which HS affects PMN degranulation. Stimulation of PMN through chemotactic receptor such as the fMLP receptor induces the activation of PKC and the ERK and p38 MAPK members that play important roles in PMN degranulation. When previously stimulated PMN are exposed to HS, they release ATP that engages P2 receptors, possibly of the P2Y2 class. These P2 receptors reinforce the signals leading to MAPK activation and increase degranulation. Released ATP, which is converted to adenosine by enzymes such as ecto-ATPases and 5'-nucleotidase (ecto-5'-NTD), activates all 4 adenosine receptor classes of PMN. A1 receptors can reduce MAPK activation and degranulation, whereas A3 receptors enhance these processes. A2 receptors seem to be unable to interfere with the activation of degranulation of previously stimulated PMN. However, as reported previously (5), A2 receptors can prevent PMN activation under temporal conditions that permit the suppressive signal of A2 receptors to intercept the activation signals that originate from the fMLP receptor.
response to PMA, suggesting that the activation of A1 receptors may block signaling events that are downstream of PKC. This inhibitory effect of A1 receptors may account, at least in part, for the observation that low concentrations of ATP had a depressive effect on degranulation, whereas the same concentrations of the nonhydrolyzable ATP analog ATPγS enhanced degranulation (Fig. 3). This was the case, although ATPγS is reportedly less potent than ATP at stimulating P2Y2 receptors (19).

Previous reports have shown that HS and ATP can augment degranulation of PMN by enhancing p38 MAPK signaling (14, 20). In the present study, we found that HS enhances degranulation via P2 and A3 receptors, both of which elicit p38 MAPK activation. We found that HS and the A3 receptor agonists selectively activated p38 but not ERK MAPK (Fig. 5C), whereas ATP was able to activate p38 as well as ERK MAPK (5). The selective activation of p38 MAPK by HS can be explained by a suppressive signal that may be introduced by the A1 receptors, which has suppressive effects on degranulation. We think this proposed mechanism could explain why HS triggers the activation of p38 MAPK and not that of ERK.

Some studies have shown that hypertonic media containing sucrose (0.40–0.45 M) can inhibit agonist-induced endocytosis of fMLP receptors (9, 12, 31) and that hypertonic saline does not alter the expression of fMLP receptors on human PMN (32). Thus it is possible that HS-induced inhibition of fMLP receptor internalization could be involved in the enhancing effects of HS on PMN responses.

However, we found that pretreatment of PMN with HS can block PMN function by preventing activation signaling through chemoattractant receptors such as the fMLP receptors. Work in our laboratory (5, 22) has previously shown that this suppressive effect of HS is largely mediated by the activation of A2 receptors, which increases intracellular cAMP level in PMN. Our group and others (7, 14, 23) have found that HS, if used with previously stimulated PMN, is not able to suppress PMN activation and that it may even have the opposite effect, namely, to increase PMN degranulation, which would aggravate tissue damage in trauma patients. In the present study, we have shown that these enhancing effects are likely due to the actions of ATP release and feedback mechanisms that involve P2 and A3 receptors. We think that our study could be useful in the development of novel hypertonic resuscitation fluids with improved clinical properties by combining HS with pharmacological adjuvants that modulate the autocrine feedback mechanisms involved in the upregulation of PMN degranulation.

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