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Hypertonic Stress Increases T Cell Interleukin-2 Expression through a Mechanism That Involves ATP Release, P2 Receptor, and p38 MAPK Activation*

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Hypertonic stress (HS) can alter the function of mammalian cells. We have reported that HS enhances differentiated responses of T cells by increasing their ability to produce interleukin (IL)-2, a finding of clinical interest because hypertonic infusions may modulate immune function in patients. HS shrinks cells and mechanically deforms membranes, which results in ATP release from many cell types. Here we investigate if ATP release is an underlying mechanism through which HS augments T cell function. We found that mechanical stress and HS induced rapid ATP release from Jurkat T cells. HS and exogenous ATP mobilized intracellular Ca^{2+}, activated p38 MAPK, and increased IL-2 expression. Ca^{2+} mobilization was attenuated in the presence of EGTA or by removal of extracellular ATP with apyrase. Adenosine did not increase IL-2 expression, as did ATP. Apyrase, inhibition of P2 receptors, or inhibition of p38 MAPK with SB203580 reduced the stimulatory effects of HS, indicating that HS enhances IL-2 expression through a mechanism that involves ATP release, P2 (perhaps P2X7) receptors, and p38 MAPK activation. We conclude that release of and response to ATP plays a key role in the mechanism through which hypertonic stress regulates the function of T cells.

Hypertonic stress (HS)[1] increases cytokine expression of immune cells and enhances T cell proliferation (1, 2). Peripheral blood mononuclear cells show increased production of IL-1α, IL-1β, IL-8, and tumor necrosis factor-α when the cells are exposed to elevated extracellular tonicity (3). Hypertonic stimulation augments the ability of T cells to produce IL-2, tumor necrosis factor-α, and lymphotoxin-β (2, 4) and restores IL-2 expression in the presence of anti-inflammatory mediators (5). Hypertonic solutions can be used to resuscitate trauma patients, who are predisposed to septic complications, and can improve T cell function after hemorrhagic shock, reducing the risk of sepsis after trauma (1, 6).

The enhancing effect of HS on IL-2 expression is paralleled by robust tyrosine phosphorylation of a number of intracellular proteins including p38 MAPK (2). This kinase is structurally related to the yeast protein HOG-1, which is part of the signaling system that allows yeast cells to regulate gene transcription in response to osmotic stress (7). In human T cells, p38 MAPK signaling is involved in the activation of IL-2 gene expression (8). Therefore, by analogy with its yeast counterpart, osmotic activation of the p38 MAPK may be involved in the mechanisms whereby HS enhances IL-2 expression of T cells.

In yeast, two osmoreceptors are known to activate HOG-1. No equivalent mammalian osmoreceptors have been identified to date, and, thus, the mechanisms whereby T cells respond to extracellular tonicity are unclear (7). HS results in cell shrinkage and mechanical deformation of the cell membrane. In a number of cell types, mechanical stress activates multiple signaling enzymes, including p38 MAPK (9, 10). Thus, the mechanisms by which these cells detect and respond to direct mechanical stimulation may be similar to those involved in the recognition of HS.

Mechanical stimulation can cause the rapid release of ATP (11–13). Once released into the extracellular environment, ATP can regulate cell function in an autocrine/paracrine manner by interacting with P2X and/or P2Y receptors that are expressed on the surface of virtually all mammalian cells (13–16). Extracellular ATP and its metabolic products, including adenosine, which acts via P1 receptors, exert a strong influence on lymphocyte function: ATP can stimulate the proliferation of mouse thymocytes, and ATP and adenosine can antagonize and/or complement T cell receptor-induced signaling, apoptosis, and thymocyte differentiation (16–18). In this study, we tested the hypothesis that ATP release in response to osmotic stimulation could be a mechanism whereby HS enhances IL-2 expression of T cells.

EXPERIMENTAL PROCEDURES

Materials—ATP and a nonhydrolyzable ATP analog, ATPγS, suramin, apyrase, adenosine deaminase, and Me_2SO were from Sigma, whereas 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 2′,3′-dialdehyde ATP (oxidized ATP, o-ATP), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and SB203580 were from Calbiochem. The Ca^{2+}-sensitive intracellular probe Fura-2 was obtained from Molecular Probes, Inc. (Eugene, OR). Antibodies to CD3 were purified from the supernatant of OKT-3 cells (clone CRL 8001) obtained from ATCC and anti-CD28 antibodies (clone CD28.2) were from BD Pharmingen (San Diego, CA). Polyclonal goat anti-P2X7 antibodies were from Santa Cruz Biotechnology, Inc. (Santa

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[1] The abbreviations and trivial names used are: HS, hypertonic stress; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); KN-62, 1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; o-ATP, oxidized ATP; PBMC, peripheral blood mononuclear cells; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; MAPK, mitogen-activated protein kinase; PHA, phytohemagglutinin; IL, interleukin.

Cruz, CA), and normal horse serum, biotinylated anti-goat antibodies, and fluorescein avidin D cell sorter grade conjugate were from Vector Laboratories (Burlingame, CA).

Cells and Cell Stimulation—Jurkat T cells (clone E6–1) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (University of California San Diego core facility, La Jolla, CA), and 10% (v/v) heat-inactivated fetal calf serum (Irvine Scientific). Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized venous blood of healthy human volunteers as described before (5).

Jurkat cells were stimulated by simultaneously activating the T cell receptor-CD3 complex and CD28 co-receptor with Dynabeads (Dynal Inc., Lake Success, NY) coated with anti-CD3 and anti-CD28 antibodies. The beads (10^7), precoated with anti-mouse IgG, were coated with anti-CD3 and anti-CD28 by incubation with 5 μg of each antibody at room temperature for 1 h. Then the beads were washed twice with RPMI with 10% fetal calf serum and resuspended at 10^7 beads/ml, and 20 μl was added to 2×10^4 Jurkat cells (10 beads/cell). The cells were incubated in a final volume of 200 μl for 20 h at 37 °C under tissue culture conditions, and IL-2 in the supernatant was determined. PBMC were suspended in the RPMI medium described above (10^5/ml) and stimulated under tissue culture conditions with 0.5 μg/ml phytohemagglutinin (PHA; Abbott) in a final volume of 200 μl for 20 h. IL-2 concentrations in the supernatants were determined with the enzyme-linked immunosorbent assay method described below.

Where indicated, cells were pretreated for 1 h at 37 °C with different agents including P1 and P2 receptor antagonists or the p38 MAPK inhibitor SB203580 (19). Cells were subjected to HS by adding appropriate volumes of RPMI containing 1 M NaCl. All materials and compounds used in these experiments were sterile and endotoxin-free.

ATP Release—ATP release from Jurkat cells was monitored in real time with cells suspended in luciferase reagent. Cells were brought to a density of 5×10^5/ml in RPMI without fetal calf serum, and 25 μl/well was transferred to a 96-well luminometer plate. Luciferase reagent in RPMI (25 μl/well) was then added (ATP Bioluminescence Assay Kit CLS II; Roche Diagnostics GmbH, Mannheim, Germany). The plate was placed in a temperature-controlled luminometer (Luminoskan; Labsystems, Helsinki, Finland), and sequential readings were taken as indicated. For quantitative ATP measurements, cells were preincubated for 3 h at 37 °C and stimulated with hypertonic saline for different periods. The cells were then placed on ice, and ATP concentrations in the supernatants were determined.

IL-2 Expression—IL-2 released into the supernatants after 20 h was measured with an enzyme-linked immunosorbent assay using monoclonal mouse anti-human IL-2 as a primary antibody (clone 5355.111) and biotinylated goat anti-human IL-2 as a secondary antibody (both from R&D Systems Inc., Minneapolis, MN), recombinant human IL-2 as a standard (Genzyme Diagnostics, Cambridge, MA), and horseradish peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA). The enzyme-linked immunosorbent assay was performed according to the recommendations provided by R&D Systems.

p38 MAPK Expression and Activation—To measure p38 MAPK activation, Jurkat cells (10^6 cells/sample) stimulated in the different experiments were placed on ice, centrifuged, resuspended in 100 μl of ice-cold SDS sample buffer containing 100 mM dithiothreitol, and lysed by boiling for 5 min. The cell lysates were separated by SDS-PAGE electrophoresis using 8–16% Tris/glycine polyacrylamide gradient gels (Novex, San Diego, CA). Lysed 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 (on Thr^{180}/Tyr^{182}), and thereby activated, form of p38 MAPK (New England Biolabs, Beverly, MA). Antibodies recognizing both active and inactive p38 MAPK were from Santa Cruz Biotechnology, and secondary antibody conjugates as well as the ECL assay kit were from Amersham Biosciences.

Intracellular Ca^{2+} Measurements—Intracellular Ca^{2+} levels were determined as described previously (20) using the fluorescent Ca^{2+} probe Fura-2 and a spectrofluorimeter (Kontron Instruments, Zurich, Switzerland). In experiments, where extracellular Ca^{2+} was chelated, EGTA (Calbiochem) was added at a final concentration of 10 mM.

P2X7 Receptor Expression—Expression of the P2X7 receptor in Jurkat cells was determined by immunoblotting of cell membrane fractions and by immunofluorescence staining. Cells were washed in ice-cold phosphate-buffered saline, resuspended in 5 ml of homogenization buffer (30 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, and 2 mM dithiothreitol, pH 7.5), homogenized with a sonicator, and centrifuged at $300 \times g$ for 5 min at 4 °C. The supernatant was centrifuged at $5{,}000 \times g$ for 10 min at 4 °C, resulting in a pellet containing a crude membrane preparation. Purified plasma membrane preparations were obtained by density gradient centrifugation of the cell homogenate using 30% Percoll at $64{,}000 \times g$ for 30 min. The crude and purified membrane preparations were boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblotting with goat anti-P2X7 antibodies (Santa Cruz Biotechnology).

For immunostaining, cells were attached to microscope slides by cytocentrifugation, fixed with chilled acetone for 10 s, and dried. Then the cells were blocked with 10% normal horse serum in phosphate-buffered saline for 20 min and incubated with 20 μg/ml goat anti-P2X7 antibody in 10% horse serum for 1 h. The cells were washed with phosphate-buffered saline, incubated for 10 min with biotinylated horse anti-goat IgG antibody in 10% horse serum, washed with phosphate-buffered saline, and incubated for another 10 min with fluorescein avidin D cell sorter grade conjugate according to the supplier's recommendations. The cells were washed five times with phosphate-buffered saline, mounted with Crystal/Mount (Biomeda Corp., Foster City, CA), and examined with a fluorescence microscope (Leica, Wetzlar, Germany). Fluorescence and modulation contrast images were acquired of cells that were stained with anti-P2X7 and of control cells that were treated identically to the antibody-stained cells, except that the primary anti-P2X7 antibody was omitted.

Statistical Analyses—Unless otherwise indicated, data are presented as mean ± S.D. Sets of data were compared with Student's t test, using $p < 0.05$ as the level of significance.

RESULTS

Mechanical Stimulation and HS Cause Rapid ATP Release—We first investigated whether mechanical stimulation causes ATP release from Jurkat cells. Cells were suspended in RPMI containing luciferase reagent, and ATP release was monitored on-line with a luminometer. Initially high luminescence readings, indicative of ATP release due to cell handling, gradually decreased, reaching base-line levels within 150 min (Fig. 1*A*, *left*). When the cells were mechanically stimulated by gentle aspiration with a pipette four times, the luminescence signal rapidly peaked within 1 min and returned toward base-line levels by 100 min. These results show that mechanical stimulation causes the release of ATP into the extracellular space. The steady decline of luminescence readings after mechanical stimulation indicates that ATP release ceases shortly after the stimulus and that released ATP is hydrolyzed, presumably by ectoapyrases, ecto-ATPases, and/or ecto-5′-nucleotidases expressed on the Jurkat cell surface (21).

Because HS causes rapid cell shrinkage, we hypothesized that the associated mechanical forces could trigger ATP release from T cells. We tested this possibility by monitoring ATP release in response to increasing levels of HS. The addition of hypertonic solutions rapidly released ATP in a concentration-dependent fashion (Fig. 1*A*, *right*). Peak luminescence signals reached a maximum within 4 min and gradually decreased to base-line readings with kinetics similar to those observed with mechanical agitation. The peak ATP signal in response to stimulation with 80 mM hypertonicity was more than 3 times as high as that observed in response to 40 mM hypertonicity. ATP release was less pronounced in response to 160 mM, suggesting that higher levels of hypertonicity may exert suppressive effects on ATP release.

The relation between HS levels and ATP release was further examined by measuring ATP release using an approach similar to that described by Yegutkin *et al.* (12), who prevented the degradation of extracellular ATP with suramin, a drug that blocks P2 receptors and also inhibits ecto-5′-nucleotidases. These experiments confirmed that exposure to HS causes a fast and dose-dependent increase in extracellular ATP. The addition of an equivalent volume of isotonic control solution caused a considerably smaller increase in extracellular ATP, which is likely the result of mechanical perturbations caused by adding this solution (Fig. 1*B*, *left*). ATP release increased with hyper-

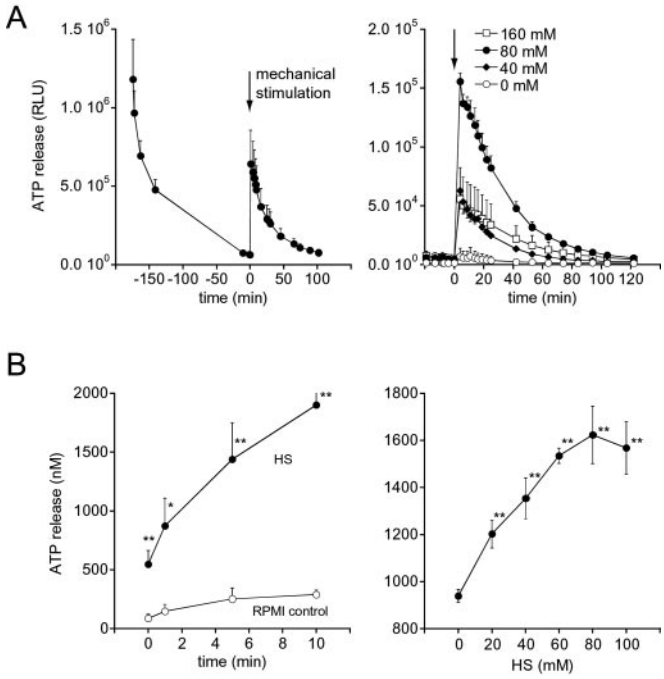

FIG. 1. **HS causes rapid ATP release and enhances IL-2 expression.** *A*, ATP release from Jurkat T cells suspended in luciferase reagent was monitored in a plate luminometer. Cells (1.25×10^4) suspended in RPMI containing luciferase reagent were pipetted into the wells of a 96-well plate and left undisturbed until luminescence readings returned to base-line levels (shown in *left panel*). At time point 0 min, the cells were mechanically agitated by gently pipetting the cell suspensions up and down four times (*left*) or exposed to osmotic stress by adding equal volumes of RPMI containing additional NaCl to increase the final tonicity of the culture medium by 0, 40, 80, or 160 mM (*right*). The results shown are representative of three experiments performed on different days, and values (relative luminescence units (*RLU*)) are expressed as the mean ± S.D. of triplicate determinations. *B*, ATP released into the culture supernatant was determined with a commercially available ATP bioluminescence assay kit. Jurkat T cells (5×10^6/ml) were allowed to rest for 3 h at 37 °C before they were osmotically stimulated with 100 mM HS (*left*) or the indicated hypertonicity levels (*right*) in the presence of 200 μM suramin to prevent ATP degradation. ATP concentrations in the culture supernatants of quadruplicate samples were determined at the indicated time points (*left*) or after 5 min (*right*). The results shown consist of the average ± S.D. of three experiments that were performed on different days. The *asterisks* indicate statistically significant differences from isotonic control values (*, $p = 0.001$; **, $p < 0.001$).

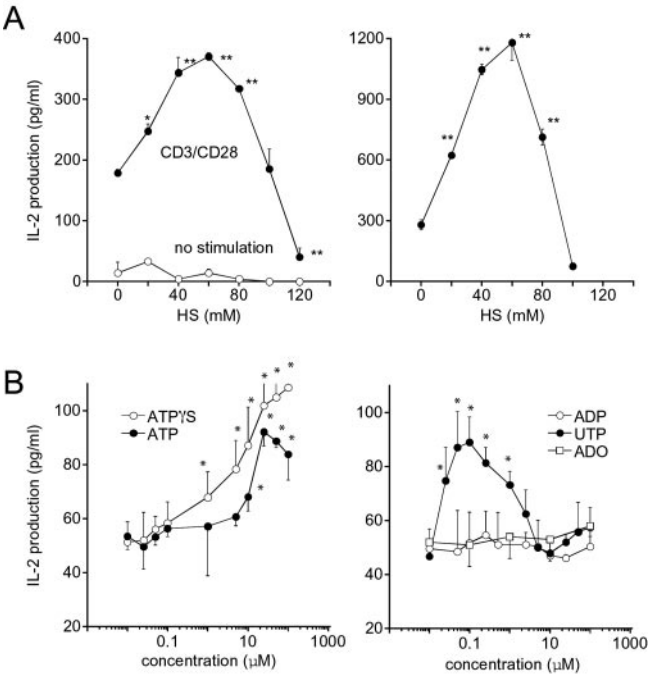

FIG. 2. **HS and ATP enhance IL-2 expression.** Jurkat cells (*A*, *left*) and isolated human PBMC (*A*, *right*) were stimulated in the presence of increasing HS concentrations with anti-CD3/CD28 antibodies or PHA, respectively, and IL-2 expression was determined after 20 h. *B*, the effects of exogenous ATP, ATPγS (*left*), UTP, ADP, and adenosine (*ADO*; *right*) on IL-2 expression of anti-CD3/CD28-stimulated Jurkat cells was determined. The results shown are representative of three individual experiments, and values are expressed as mean ± S.D. of triplicate determinations; *asterisks* indicate statistically significant differences from control values in the absence of HS or nucleotides (*, $p < 0.05$; **, $p \leq 0.01$).

tonicity levels peaking at 80 mM HS (Fig. 1*B*, *right*).

HS and Extracellular ATP Co-stimulate IL-2 Expression—The similarities between ATP release and other T cell responses to HS suggested that ATP release could be involved in the mechanisms whereby HS enhances IL-2 expression. To test this possibility, Jurkat cells were stimulated with antibodies to CD3 and CD28 in the presence of increasing levels of HS or exogenous ATP, and IL-2 production was measured 20 h later. HS at concentrations up to 60 mM increased IL-2 expression of Jurkat cells and isolated PBMC, whereas higher levels of HS suppressed IL-2 production (Fig. 2*A*). Similar to HS, the addition of exogenous ATP significantly increased IL-2 expression of Jurkat cells in a concentration-dependent fashion (Fig. 2*B*, *left*). ATP concentrations of 10–40 μM significantly increased IL-2 expression. As with increasing levels of HS, higher concentrations of ATP progressively reduced the enhancement of IL-2 expression. The nonhydrolyzable ATP analog ATPγS also enhanced IL-2 expression, doubling IL-2 expression at concentrations of ≥20 μM (Fig. 2*B*, *left*). In contrast to the results with ATP and HS, we observed no bimodal effect at higher concentrations of ATPγS. UTP at concentrations between 0.02 and 1 μM also enhanced IL-2 expression, whereas ADP and adenosine (*ADO*) had no discernible effects (Fig. 2*B*, *right*). These data demonstrate that certain nucleotides, but not ADP and adenosine, can co-stimulate IL-2 expression, supporting the notion that activation of specific nucleotide receptors may contribute to the regulation of T cell function by HS.

HS and ATP Cause Ca^{2+} Mobilization—Extracellular nucleotides, such as ATP, act via plasma membrane P2Y and P2X receptors (14, 16, 18). Activation of both receptor types results in intracellular Ca^{2+} mobilization. To test whether exposure of Jurkat cells to HS or ATP could elicit intracellular Ca^{2+} mobilization, we loaded Jurkat cells with the Ca^{2+} probe Fura-2 and assessed [Ca^{2+}] in response to HS or the addition of ATP. HS rapidly and concentration-dependently increased intracellular [Ca^{2+}] in Jurkat cells (Fig. 3*A*, *left*). This response was blunted when extracellular ATP was hydrolyzed with apyrase or when extracellular Ca^{2+} was bound with EGTA (Fig. 3*A*, *right*). The peak Ca^{2+} concentrations corresponded to the applied levels of HS and were consistently reduced by apyrase (Fig. 3*B*). Exogenous ATP and ATPγS mobilized Ca^{2+} over a similar time course (Fig. 3*C*). Whereas 0.5–1.0 mM ATP was required to mobilize calcium, ~100-fold lower concentrations of ATPγS increased intracellular [Ca^{2+}], suggesting that much of the added ATP may be hydrolyzed before it can activate P2 receptors on the T cell membrane. Together, these findings suggest that HS-promoted ATP release can trigger secondary signaling events, such as Ca^{2+} mobilization via activation of purinergic receptors, which may be involved in the up-regulation of IL-2 expression by HS.

HS Enhances IL-2 Expression via Released ATP—As a further test of the idea that ATP released in response to HS augments the ability of CD3/CD28-stimulated T cells to ex-

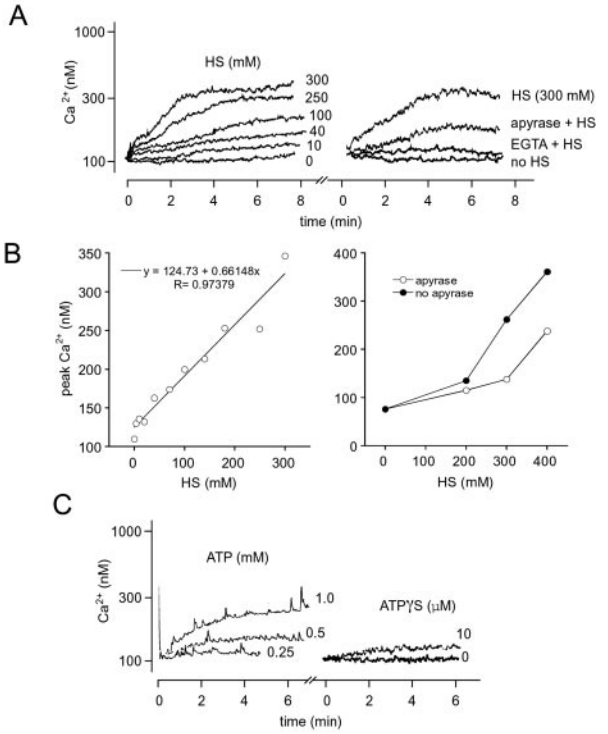

FIG. 3. **HS and ATP mobilize intracellular Ca^{2+}.** Jurkat T cells were loaded with the Ca^{2+} probe Fura-2, exposed to the indicated levels of HS (*A*, *left panel*) or to HS (300 mM) in the presence or absence of apyrase or EGTA (*A*, *right panel*), and Ca^{2+} mobilization was recorded. Peak intracellular Ca^{2+} levels in the presence or absence of apyrase were plotted *versus* the corresponding HS levels (*B*). For comparison, Ca^{2+} mobilization in response to stimulation with the indicated concentrations of ATP or ATPγS was determined (*C*). Data shown are representative of three different experiments with similar results.

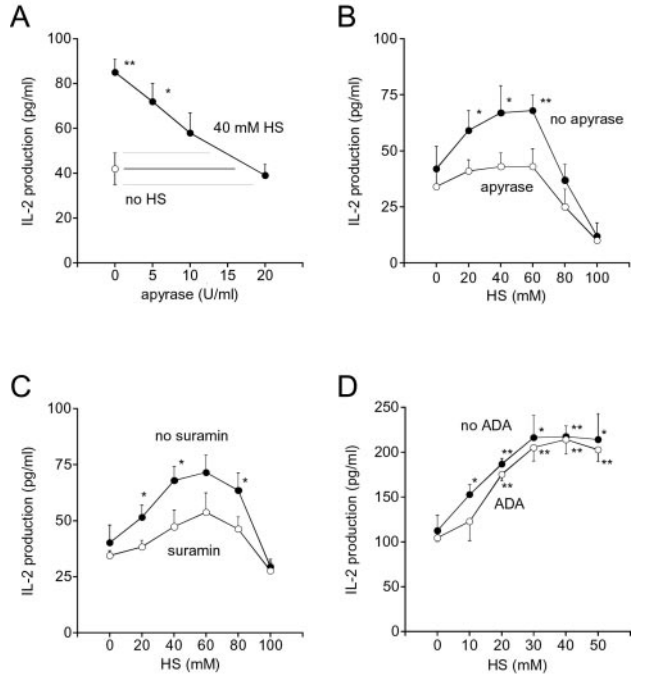

FIG. 4. **Extracellular ATP is required for the enhancement of IL-2 expression by HS.** Jurkat cells were stimulated with bead-bound anti-CD3/CD28 antibodies in the presence of the indicated HS levels and of increasing concentrations of apyrase (*A*) or of 20 units/ml apyrase (*B*) to break down released ATP, in the presence (*empty symbols*) or absence (*filled symbols*) of 200 μM suramin to block P2 receptors (*C*) or in the presence (*empty symbols*) or absence (*filled symbols*) of 1 unit/ml adenosine deaminase (*ADA*). IL-2 production was determined as described above. Results are representative of three individual experiments, and data are means ± S.D. of triplicate determinations. *Asterisks* indicate significant differences between treated *versus* untreated samples (*B* and *C*) and HS *versus* isotonic controls (*A*) (*, $p < 0.05$; **, $p \leq 0.01$).

press IL-2, we exposed CD3/CD28-stimulated Jurkat cells to HS in the presence of apyrase, an enzyme that scavenges extracellular ATP (22). Apyrase concentration-dependently reduced the co-stimulatory effects of HS (Fig. 4*A*). At a concentration of 20 units/ml, apyrase almost completely prevented the enhancement of IL-2 expression by increasing levels of HS (Fig. 4*B*). These data are consistent with the idea that ATP released into the extracellular space plays a critical role in the events that lead to enhanced IL-2 expression in the presence of HS. As a second approach to examine the role of ATP release in the enhancement of IL-2 expression, we used the P2 receptor antagonist, suramin, which nonselectively blocks P2 receptors and, in addition, as noted above, inhibits ecto-ATPases (14). Suramin significantly reduced the enhancing effects of HS on IL-2 production (Fig. 4*C*). ATP can be hydrolyzed to adenosine by ectoenzymes (17), and we investigated whether adenosine might be involved in the enhancing effects of HS on IL-2 production. The addition of adenosine deaminase to hydrolyze adenosine did not alter the effect of HS on IL-2 expression (Fig. 4*D*). This suggests that ATP but not adenosine is responsible for the enhancing effects of HS.

Role of P2X7 Receptors—Stimulation of P2X7 receptors has been reported to increase intracellular [Ca^{2+}] and to synergize T cell proliferation in response to mitogens, such as PHA (18). We used two approaches, immunoblotting of membranes (Fig. 5*A*) and immune fluorescence analysis of cells (Fig. 5*B*), and found that Jurkat T cells express P2X7 receptors. In addition, we tested the role of P2X7 in the response of T cells to HS using the P2 receptor antagonists KN-62, o-ATP, and PPADS, all of which antagonize P2X7 receptors (14). Jurkat T cells or isolated PBMC were treated with increasing concentrations of these antagonists for 20 min, exposed to 40 mM HS, and stimulated with CD3/CD28 or PHA, respectively. With IC_{50} values of about 0.1 μM, KN-62 was the most potent of the three compounds in reducing the effect of HS on IL-2 production (Fig. 5*C*). PPADS and o-ATP also reduced IL-2 expression, although higher antagonist concentrations were needed; both PPADS and o-ATP suppressed IL-2 expression of PBMC beyond the IL-2 expression level reached under isotonic conditions (*dashed line*). PBMC preparations consist not only of T cells but also of B cells and monocytes; the antagonists may affect P2 receptors in these other cells that serve as accessory cells to T cells. The inhibitory effect by o-ATP was observed with both Jurkat and peripheral T cells, suggesting that o-ATP may not only affect the P2X7 receptor but also perhaps other P2 receptors that influence T cell activation under normal, isotonic conditions.

ATP, Like HS, Activates p38 MAPK—HS is known to activate p38 MAPK in T cells (2). Because p38 MAPK signaling targets several nuclear factors that may be responsible for T cell gene regulation, we tested whether p38 MAPK contributes to the mechanisms through which HS enhances IL-2 expression. We thus compared the roles of p38 MAPK signaling in response to stimulation with ATP or HS. HS levels of 20, 40, and 100 mM activated p38 MAPK in a concentration- and time-dependent fashion (Fig. 6*A*). p38 MAPK activation was detectable at 40 and 100 mM hypertonicity by 3 min, but the highest activation levels were observed 30–60 min after stimulation with HS. Stimulation of T cells with ATP caused a less pronounced activation of p38 MAPK with a peak at 1 min (Fig. 6*B*). ATP concentrations between 0.01 and 1 μM were most effective in the stimulation of p38 MAPK activation (Fig. 6*C*).

These data show that extracellular ATP can trigger p38 MAPK activation and that ATP release in response to hypertonic stimulation of T cells may contribute to HS-promoted activation of p38 MAPK. A reason for the different kinetics and amplitudes of p38 MAPK stimulation by HS and ATP may be the prolonged nature of hypertonic stimulation compared with stimulation with ATP.

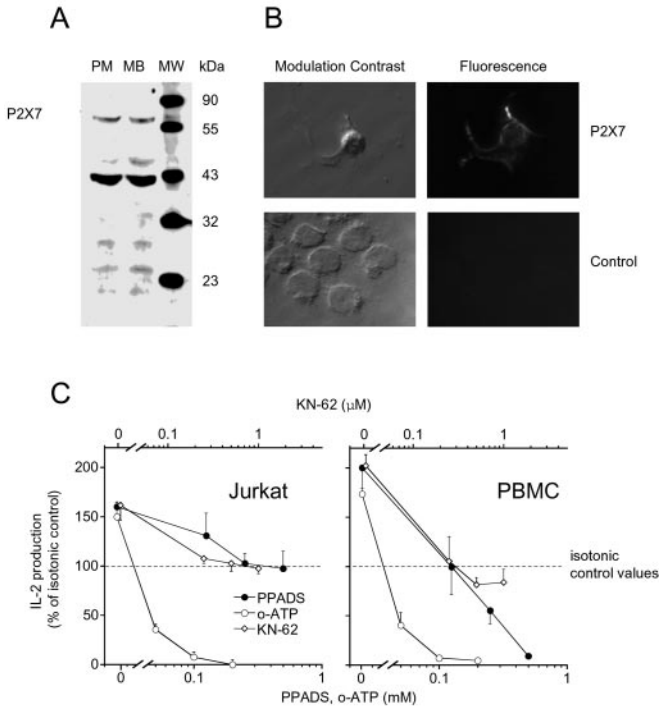

FIG. 5. **P2X7 receptor expression and action in Jurkat cells and PBMC.** Jurkat cells express P2X7 as shown by immunoblotting of crude membranes (*MB*) and purified membranes (*PM*) of Jurkat cells (*A*) and in the fluorescence image of Jurkat cells stained with P2X7 antibodies (*B*, *upper panels*) or control cells that were stained without P2X7 antibodies (*B*, *lower panel*). Jurkat cells (*C*, *left*) or isolated human PBMC (*C*, *right*) were pretreated for 20 min with the indicated concentration of the P2X7 antagonist PPADS, o-ATP, or KN-62, stimulated with 40 mM HS and anti-CD3/CD28 or PHA, respectively. IL-2 production was determined as in Fig. 2 and is expressed as a percentage of the IL-2 production of control cells that were stimulated in the absence of HS.

p38 MAPK Is an Important Element in the Mechanism whereby HS Enhances IL-2 Expression—The findings above suggest that p38 MAPK signaling may contribute to the enhancement of IL-2 expression by HS and ATP. We thus inhibited p38 MAPK with the inhibitor SB203580 and investigated whether this would prevent the enhancing effects of HS on CD3/CD28-stimulated IL-2 expression. SB203580 concentration-dependently reduced the enhancement of IL-2 expression by 40 mM hypertonicity. Increasing concentrations of SB203580 also reduced IL-2 expression of cells stimulated in the absence of HS (Fig. 7). The latter finding corresponds to earlier reports that suggested a role of p38 MAPK in the activation of IL-2 expression of Jurkat cells (8). SB203580 completely abrogated the enhancement of IL-2 expression by a wide range of HS conditions (Fig. 7). These results imply that p38 MAPK plays a major role in the enhancement of IL-2 expression by HS.

DISCUSSION

Under normal physiological conditions, plasma tonicity can vary significantly as a consequence of water loss and salt uptake. Plasma tonicity is affected by age (23), climate, and seasonal changes (24) and by activities such as water/salt uptake and physical exercise (25, 26), the latter of which can affect the ratio of T cell subsets in the circulation (25). Whereas these external influences can alter plasma tonicity, even more extreme differences in extracellular tonicity occur within the organs of the gastrointestinal and urinary tract. It is possible that elevated tonicity could increase the level of vigilance of the immune cells patrolling these organs.

Aside from HS, other conditions leading to cellular distress can result in ATP release (*e.g.* tissue injury or hypoxia during hemorrhagic shock) (27). ATP and its metabolites may allow the host to better cope with injury by increasing T cell function. Indeed, administration of ATP-$MgCl_2$ or inhibition of adenosine kinase has been shown to improve survival after hemor-

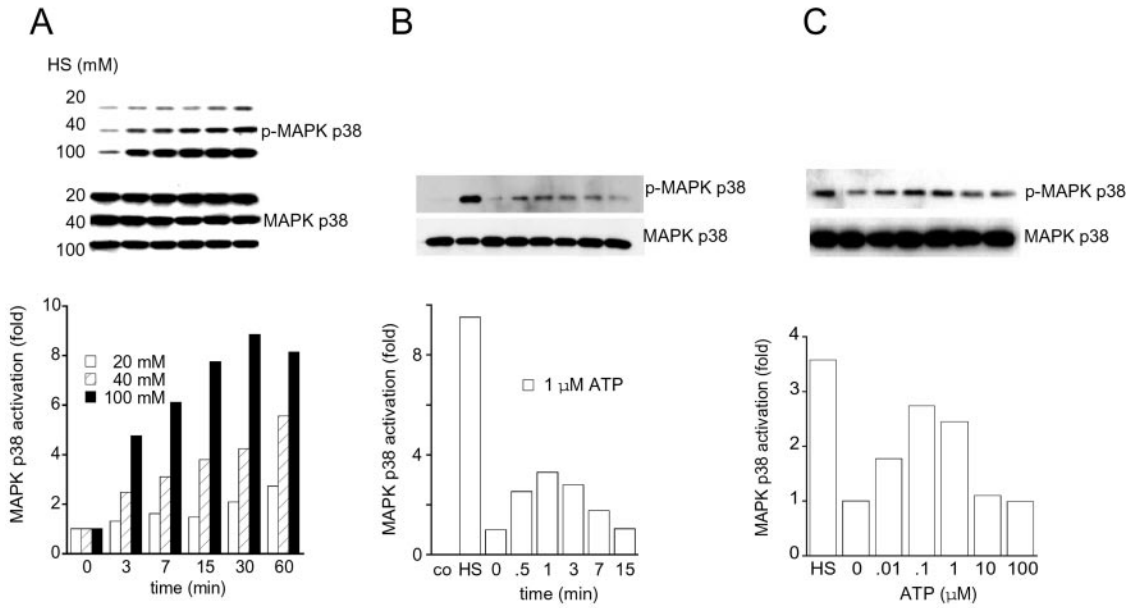

FIG. 6. **HS and ATP activate p38 MAPK in Jurkat cells.** Timing of p38 MAPK activation by HS (*A*) and ATP (*B*) and the dose response to ATP stimulation were determined by stimulating Jurkat cells with the indicated levels of HS or ATP for different periods or for 5 min (*C*). p38 MAPK activation was determined by immunoblotting with phosphospecific anti-p38 MAPK antibodies (*upper lanes*). To control for equal protein loading, membranes were reprobed with antibodies recognizing active and inactive p38 MAPK (*lower lanes*). Band intensities were analyzed, and ratios between activated and total p38 MAPK were used to calculate p38 MAPK activation. In *B* and *C*, cells were stimulated with 100 mM HS as a positive controls. The data shown are representative of three experiments with similar results.

rhagic shock and sepsis and to protect lungs from ischemia reperfusion injury (27–29). Because of its protective effects, the ATP metabolite, adenosine, has been termed the "retaliatory metabolite in ischemia-reperfusion" (27).

In trauma patients, hypertonic solutions can be used to rapidly restore lost blood volume (6). The infusion of these solutions transiently increases plasma tonicity to levels that we report here to cause substantial ATP release from T cells. As observed with ATP-$MgCl_2$ administration, hypertonic solutions can improve the outcome after hemorrhagic shock and sepsis in both animal models and patients (1, 6). The present study suggests that ATP release in response to hypertonic solutions could mediate these beneficial *in vivo* effects by enhancing the function of T cells.

It has long been recognized that cells release ATP under pathological conditions that involve hypoxia, cell damage, loss of cell viability, and cytolysis (14, 15). More recently, it has been noted that healthy cells release ATP under normal physiological conditions. Mechanical and osmotic stimulation have been shown to cause ATP release from several different cell types including endothelial and epithelial cells, fibroblasts, hepatocytes, and vascular smooth muscle cells (*e.g.* Refs. 12, 13, 30, and 31). There are at least three possible mechanisms for the regulated release of nucleotides under physiological conditions: exocytosis of ATP-filled vesicles, conductive transport through membrane ion channels, and facilitated diffusion by nucleotide-specific transporters. However, work to date has not precisely resolved which mechanism is operative (22, 30).

The present study demonstrates for the first time that mechanical agitation and exposure to HS stimulate Jurkat T lymphocytes to release ATP in a manner that correlates with the level of hypertonicity to which the cells are exposed (Fig. 1). Similar ATP release patterns have been reported in response to direct mechanical stimulation of endothelial and vascular smooth muscle cells (13, 32). Exposure of mammalian cells to HS causes rapid cell shrinkage, which is accompanied by membrane deformation. Modest mechanical forces of hemodynamic origin and forces associated with increased perfusion flow rates have been shown to trigger ATP release from vascular smooth muscle and endothelial cells (15, 32, 33). Mechanical and hypertonic stimulation of T cells resulted in a rapid ATP release followed by a decrease toward base-line levels (Fig. 1). This decrease of extracellular ATP is presumed to occur via ATP hydrolysis by ecto-ATPases and ecto-5′-nucleotidases, such as CD39 and CD73, which may be expressed on the T cell membrane (18, 21). Other pathways for metabolism of extracellular nucleotides have been identified recently (*e.g.* Ref. 34).

As with HS, the addition of exogenous ATP, its nonhydrolyzable analog ATPγS, and UTP significantly increased the ability of Jurkat cells to produce IL-2, and the addition of apyrase blunted this response (Figs. 2 and 4). The results demonstrate that ATP release in response to osmotic stimulation can enhance IL-2 expression in an autocrine/paracrine fashion that probably involves P2 receptors expressed on the T cell surface. The ATP product adenosine (*ADO*) did not influence IL-2 expression (Fig. 2*B*), and adenosine deaminase, a scavenger of adenosine, did not reduce the enhancing effect of HS on IL-2 production (Fig. 4*D*), suggesting that adenosine and the adenosine or P1 receptors are not involved in the regulation of T cell function by HS.

Based on their molecular structures, P2 receptors are divided into two subfamilies: the G protein-coupled heptahelical P2Y receptors and P2X receptors that are ligand-gated ion channels. Of the mammalian P2 receptors that have been cloned to date (14), only P2X-like receptors (P2X1, P2X4, and P2X7) seem to be expressed by human T lymphocytes (18). We found that Jurkat cells express P2X7 and that P2X7-specific antagonists can attenuate the enhancing effect of HS on IL-2 expression (Fig. 5). These observations suggest that P2X7 may play an important role in the response of T cells to HS.

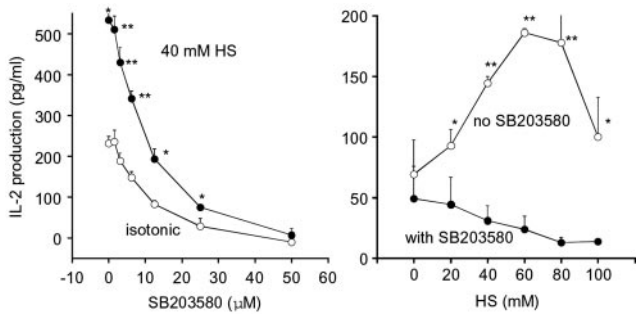

FIG. 7. **Osmotic regulation of IL-2 expression involves ATP release and p38 MAPK.** Jurkat T cells were treated for 1 h at 37 °C with either the indicated doses of the p38 MAPK inhibitor SB203580 (*left*), 20 μM SB203580, or Me_2SO vehicle only (no SB203580) (*right*). The cells were then stimulated via CD3/CD28 in the absence or presence of HS as indicated, and IL-2 production was determined. Data are representative of three different experiments and expressed as mean ± S.D. (*, $p < 0.03$; **, $p < 0.001$, $n = 3$).

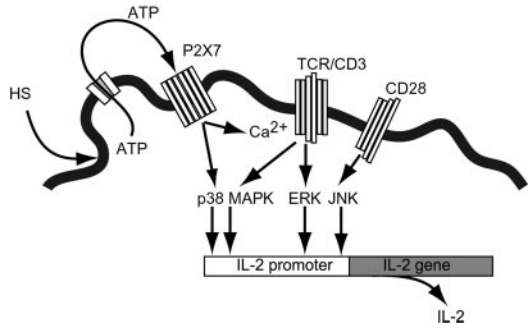

FIG. 8. **Proposed mechanism of hypertonic regulation of IL-2 expression.** We propose that HS enhances IL-2 expression of T cells via osmotic cell shrinkage, membrane deformation, ATP release into the extracellular space, the activation of P2X7 by ATP, and subsequent Ca^{2+} mobilization and p38 MAPK activation. These signals then increase IL-2 gene transcription and expression.

HS triggered robust Ca^{2+} mobilization at HS levels that increased extracellular ATP concentrations to ~2 μM (Fig. 1*B*). In comparison, much higher concentrations of exogenous ATP were required for equivalent increases of intracellular Ca^{2+} levels (Fig. 3) or p38 MAPK activation (Fig. 6). This discrepancy could be due to differences of the ATP concentrations in proximity to the T cell membrane compared with the bulk media. Extracellular nucleotides can be hydrolyzed by ecto-ATPases and ecto-5′-nucleotidases that exist on the plasma membrane of cells (21, 34); our data (Fig. 1) suggest the presence of such enzymes on Jurkat cells. ATP released in response to hypertonic stimulation is likely to achieve its highest concentrations in close proximity to the plasma membrane (*i.e.* adjacent to P2 receptors) (35). This explanation is consistent with our observation that nonhydrolyzable ATPγS was about 50 times as effective as ATP in triggering Ca^{2+} mobilization in Jurkat cells. Mechanical stimulation of other cell types causes ATP release and the propagation of Ca^{2+} waves along monolayers, indicating that ATP-induced Ca^{2+} signaling can serve as a means of communication between neighboring cells (36). Our data suggest that HS-induced ATP release may also serve as messenger between osmotically stressed cells.

p38 MAPK is activated by cellular stresses, inflammatory cytokines, lipopolysaccharide, and G protein-coupled receptors (37, 38). Activated p38 MAPK in turn mediates cytokine pro-

duction and other stress responses (19, 38). p38 MAPK is also activated by mechanical stimulation in a number of cell types (*e.g.* Refs. 39 and 40). Mechanical stimulation of vascular smooth muscle cells recently has been shown to activate p38 MAPK through a mechanism that involves ATP release and a feedback mechanism involving extracellular ATP and adenosine (32). Our data suggest that a similar feedback mechanism may be involved in the response of Jurkat T cells to HS (Fig. 6). Inhibition of p38 MAPK with SB203580 prevented the enhancement of IL-2 expression by HS, consistent with the idea that p38 MAPK signaling plays a critical role in the response of T cells to HS.

Overall, our results suggest a regulatory pathway where HS causes the release of ATP. ATP in turn activates P2 receptors that increase Ca^{2+} influx and the activation of p38 MAPK. Finally, p38 MAPK exerts a co-stimulatory signal that enhances IL-2 expression of CD3/CD28-stimulated T cells, thereby enhancing T cell function (Fig. 8). Existence of this pathway identifies new targets for the modulation of T cell function.

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Hypertonic Stress Increases T Cell Interleukin-2 Expression through a Mechanism That Involves ATP Release, P2 Receptor, and p38 MAPK Activation

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