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Shockwaves increase T-cell proliferation and IL-2 expression through ATP release, P2X7 receptors, and FAK activation

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Yu TC, Junger WG, Yuan CJ, Jin A, Zhao Y, Zheng XQ, Zeng YJ, Liu JG. Shockwaves increase T-cell proliferation and IL-2 expression through ATP release, P2X7 receptors, and FAK activation. *Am J Physiol Cell Physiol* 298: C457–C464, 2010. First published November 4, 2009; doi:10.1152/ajpcell.00342.2009.—Shockwaves elicited by transient pressure disturbances are used to treat musculoskeletal disorders. Previous research has shown that shockwave treatment affects T-cell function, enhancing T-cell proliferation and IL-2 expression by activating p38 mitogen-activated protein kinase (MAPK) signaling. Here we investigated the signaling pathway by which shockwaves mediate p38 MAPK phosphorylation. We found that shockwaves at an intensity of 0.18 mJ/mm^2 induce the release of extracellular ATP from human Jurkat T-cells at least in part by affecting cell viability. ATP released into the extracellular space stimulates P2X7-type purinergic receptors that induce the activation of p38 MAPK and of focal adhesion kinase (FAK) by phosphorylation on residues Tyr397 and Tyr576/577. Elimination of released ATP with apyrase or inhibition of P2X7 receptors with the antagonists KN-62 or suramin significantly weakens FAK phosphorylation, p38 MAPK activation, IL-2 expression, and T-cell proliferation. Conversely, addition of exogenous ATP causes phosphorylation of FAK and p38 MAPK. Silencing of FAK expression also reduces these cell responses to shockwave treatment. We conclude that shockwaves enhance p38 MAPK activation, IL-2 expression, and T-cell proliferation via the release of cellular ATP and feedback mechanisms that involve P2X7 receptor activation and FAK phosphorylation.

p38 mitogen-activated protein kinase; ATP; focal adhesion kinase

THE CONCEPT that physical forces such as pressure, shear, and cell deformation can modulate cellular functions has been well established (6, 12, 15, 17, 35, 38, 44, 46–51). Shockwaves are elicited by transient pressure disturbances, characterized by tensile waves with high positive pressures and rise times of $<$10 ns (49). Shockwaves exert mechanical stimulation that elicits various biological effects (49). Shockwaves are widely used in orthopedic procedures, for example, to treat insertional tendinopathies (enthesiopathies) and delayed union and nonunion fractures. These effects are thought to be related to specific effects of shockwaves on bone cells and immune cells (49). A number of studies have focused on the cellular mechanisms by which shockwave treatment affects musculoskeletal disorders (47–49). Our group has found that shockwaves enhance the proliferation and IL-2 expression of T-cells through phosphorylation of p38 mitogen-activated protein kinase (MAPK) (47, 48). However, the upstream mechanisms by which shockwaves elicit these cell responses have remained unclear.

Human Jurkat T-cells and neutrophils release ATP in response to localized cell stimulation at the immune synapse facing accessory cells or at the leading edge of neutrophils facing chemotactic agents (7, 45). The released ATP plays a key role in amplifying T-cell activation and chemotactic gradient signals that facilitate T-cell responses or cell polarization and directed migration through feedback mechanisms that involve P2X- and P2Y-type nucleotide receptors (7, 45). In addition to regulated ATP release in response to cell stimulation, mechanical stress also causes ATP release (14, 29). Because shockwave treatment elicits mechanical stress and cell deformation, we investigated in the current study whether augmented IL-2 production and T-cell proliferation in response to shockwave treatment involve ATP release and autocrine/paracrine feedback via P2 receptors.

Cell polarization and migration require the activation of focal adhesion kinase (FAK), also known as PTK2 protein tyrosine kinase 2 (PTK2) (25, 26). FAK activation occurs through phosphorylation at the Tyr397 and Tyr576/577 residues (5, 26). In this study we investigated whether shockwave treatment induces FAK activation and how this protein interacts with the signaling processes involved in T-cell responses to shockwave treatment.

MATERIALS AND METHODS

Materials. Apyrase and dimethylsulfoxide were from Sigma (St. Louis, MO), whereas 1-[*N*, *O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), suramin, phytohemagglutinin (PHA), anti-phospho-Tyr397-FAK antibodies, anti-phospho-Tyr576/577-FAK antibodies, pan-FAK antibodies, and SB203580 were from BioSource International (Camarillo, CA). DMEM, heat-inactivated fetal bovine serum (FBS), and RPMI-1640 were from GIBCO (Invitrogen, Tulsa, OK). Oligofectamine was from Life Technologies (Gaithersburg, MD) and a KDE-2001 Extracorporeal Shockwave Lithotripter was from Beijing Zhongke Jian An Meditech (Beijing, China). A membrane hydrophone was purchased from Precision Acoustics (Dorchester, Dorset, UK). Polystyrene round-bottom tubes were from Falcon Becton-Dickson (Franklin Lakes, NJ). Ultrasound transmission gel was purchased from Pharmaceutical Innovations (Newark, NJ). An ATP Bioluminescence Assay Kit was purchased from Calbiochem (San Diego, CA), a temperature-controlled Luminoskan luminometer was purchased from Labsystems (Helsinki, Finland) and a PhosphoPlus p38 MAP kinase Antibody Kit was obtained from Cell Signaling Technology (Boston, MA). Tris-glycine polyacrylamide gradient gels were from Novex (San Diego, CA), polyvi-

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nylidene difluoride (PVDF) membranes (Immobilon-P) were purchased from Millipore (Bedford, MA), and LumiGLO chemiluminescent reagent was from Cell Signaling Technology.

Cells. Jurkat T-cells (clone E6-1) were obtained from the Shanghai Institute of Cell Biology (Cell Bank, Chinese Academy of Sciences, Shanghai, China) and maintained in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (vol/vol) heat-inactivated FBS. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood of healthy human volunteers and collected by Ficoll-Paque density gradient centrifugation ($d = 1.007$ g/ml, Amersham Biosciences).

Shockwave treatment. A KDE-2001 Extracorporeal Shockwave Lithotripter was employed for all studies. Shockwaves were generated by underwater spark discharge from an electrode located at *focus 1* of a hemiellipsoid immersed in degassed water, jacketed with a ultraviolet light (UV)-resistant outer membrane (Fig. 1). The propagation waves were focused to *focus 2* of the ellipsoid where the center of test tubes containing target cells was affixed as shown in Fig. 1. Low-density shockwaves (LDSWs) at a frequency of 50 Hz were generated at a generator voltage of 7 kV and a capacitance of 0.3 μF. The positive pressure of the shockwaves generated was determined to be 23 ± 1.4 MPa ($n = 3$) as measured with a PA membrane hydrophone according to International Electrotechnical Commission (IEC) guideline 61846 (IEC 1998, Chicago, IL; IEC 61846:1998, Ultrasonics-Pressure pulse lithotripters-Characteristics of fields) (47). The energy flux density; i.e., derived pulse intensity integral at the wave focus, was 0.18 ± 0.01 mJ/mm^2 (mean value ± SD; $n = 3$) as calculated from the waveform in the focus according to IEC 61846 guidelines.

Cells (10^6 cells/ml) were suspended in a 2-ml polystyrene round-bottom tube containing 0.5 ml RPMI-1640, pH 7.4. The cells were exposed to 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, or 600 LDSW impulses at an energy flux density of 0.18 mJ/mm^2. The duration of the LDSW treatment was 10–20 min depending on the number of impulses applied. An ultrasound transmission gel was used as contact medium between the apparatus and the target test tube. The apparatus was covered with an UV-resistant membrane to avoid UV light damage to the environment and the cells. After the shockwave treatment, cell numbers and viability were determined with a hemocytometer and trypan blue dye exclusion assay.

Cell stimulation with PHA or anti-CD3 and anti-CD28 antibodies. Jurkat cells were stimulated by simultaneously activating the T-cell receptor/CD3 complex and CD28 coreceptor with Dynabeads (Dynal, Lake Success, NY). These beads (10^7) were precoated with anti-mouse IgG and incubated with 5 μg anti-CD3 and anti-CD28 antibodies at room temperature for 1 h (24). The beads were then washed twice with RPMI containing 10% FBS and resuspended at a concentration of 10^7 beads/ml. Of this suspension, 20 μl were 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. PBMCs were suspended in RPMI-1640 medium (10^6 cells/ml) and stimulated under tissue culture conditions with a suboptimal dose of PHA (1 μg/ml) in a final assay volume of 200 μl. Dose-response curves (data not shown) indicated optimal lymphoproliferation in response to 5 μg/ml PHA. IL-2 concentrations in the supernatants were determined 20 h after stimulation, and T-cell proliferation was assessed 48 h after stimulation using the methods described below.

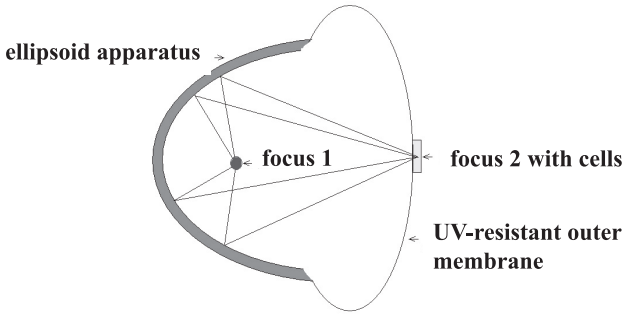

Fig. 1. Schematic diagram of shockwave apparatus. Shockwaves are generated by a KDE-2001 Extracorporeal Shockwave Lithotripter, which elicits spark discharge underwater from an electrode (*focus 1*). Degassed water is jacketed with an ultraviolet light (UV)-resistant outer membrane. The shockwaves are reflected in an ellipsoid apparatus and focused into the target tube containing experimental cells located at *focus 2*.

Cell viability assay. Cell viability was assayed using trypan blue dye exclusion (34). After Jurkat T-cells (10^6/ml) were treated as described below, the cells were cultured in 1 ml RPMI-1640 using 12-well plates. Cell viability was determined immediately (i.e., within 5 min after plating), 24 h after culture, or after the periods described below. Briefly, 20-μl aliquots of these cell suspensions were mixed with 20 μl of a 0.4% trypan blue solution (GIBCO, Invitrogen) and incubated for 3 min at room temperature. Then a drop of the trypan blue-cell mixture was applied to a hemocytometer (Haimen Tianlong Scientific Instruments, Jiangsu, China). The hemocytometer was placed onto the stage of a binocular microscope and unstained (viable) and stained (nonviable) cells were separately counted.

ATP release assay. ATP release into the culture supernatant of Jurkat T-cells was determined with a commercially available ATP Bioluminescence Assay Kit. The cells were adjusted to a density of 5×10^6/ml in RPMI without FBS and allowed to rest for 3 h at 37°C. The cells were then exposed to LDSWs in the presence or absence of 100 μM suramin, which blocks hydrolysis of extracellular ATP at this concentration (43). Cells were placed on ice and centrifuged at 12,000 *g* for 5 min, and supernatants (50 μl/well) were transferred to a 96-well plate. Luciferase reagent dissolved in RPMI was then added at a volume of 50 μl/well, the plate was placed in a temperature-controlled luminometer, and ATP concentrations were determined based on luminescence signals obtained with ATP standard solutions of known concentrations. Results shown are representative of three different experiments and expressed as means ± SD.

Transfection. Jurkat cells were transfected with small interfering RNA (siRNA) to block FAK expression. A FAK-specific siRNA construct with a previously published sequence (41) and a nontargeting control siRNA construct were obtained from Dharmacon (Lafayette, CO). The siRNA constructs were introduced using Oligofectamine reagent (Life Technologies) as per manufacturer's instructions. Both FAK-specific siRNA duplexes and nontargeting control siRNA were transfected at final concentrations of 140 nM. Briefly, oligofectamine was mixed gently, diluted in serum-free medium, and incubated for 10 min at room temperature. FAK-specific siRNA or nontargeting control siRNA was mixed with the diluted oligofectamine reagent. The mixture was incubated for 20 min at room temperature. While complexes formed, the growth medium was removed from the cells, and the cells were washed once with serum-free medium. Then the cells were incubated with FAK-specific siRNA or nontargeting control siRNA in serum-free medium. The cells were used after 24 h in Western blot assays to control for effective downregulation of FAK expression and to determine shockwave-induced p38 MAPK and FAK phosphorylation.

T-cell proliferation assay. PBMCs were exposed to low-density shockwaves at 0.18 mJ/mm^2 with 0, 50, 100, 150, 200, 250, 300, or 350 impulses. The cells were then stimulated or not with 1 μg/ml PHA, incubated at 37°C in humidified air with 5% CO_2 for 48 h, pulsed with 1 μCi/well [*methyl*-^{3}H]thymidine ([^{3}H]TdR; Carbone Scientific, Chancery Lane, London, UK), and incubated for another 8 h. Subsequently, the cells were harvested onto glass fiber filter discs using a PHD cell harvester (Cambridge Technology, Cambridge, MA). The filter discs were transferred into scintillation vials, dried overnight, and covered with 4 ml/vial scintillation fluid (ScintiVerse BD. Fisher Scientific, Fair Lawn, NJ). Incorporated thymidine was counted with a liquid scintillation counter (Tri-Crab 2100TR; Pack-

ard, Avondale, PA). Values were expressed as means ± SD of triplicate determinations.

IL-2 expression. An enzyme-linked immunosorbent assay (ELISA) was performed as per the manufacturer's instructions to detect IL-2 in the supernatant. Monoclonal mouse anti-human IL-2 (clone 5355.111) and biotinylated goat anti-human IL-2 antibodies were from R&D Systems (Minneapolis, MN). Recombinant human IL-2 was from Genzyme Diagnostics (Cambridge, MA), and horseradish peroxidase-conjugated streptavidin was from Zymed Laboratories (San Francisco, CA).

Western blot analysis. The phosphorylation of FAK on the Tyr397 or Tyr576/577 residues and of p38 MAPK on Thy180/Tyr182 was measured with anti-phospho-FAK antibodies and PhosphoPlus p38 MAPK antibodies. To investigate the roles of ATP release and P2 receptors in the phosphorylation of FAK or p38 MAPK, Jurkat T-cells were pretreated with apyrase or P2 receptor inhibitors for 1 h at 37°C and subjected to LDSW treatment, and FAK and p38 MAPK phosphorylation was determined. Briefly, cells (10^6/sample) were subjected to LDSW treatment as described above, cultured in 1 ml RPMI-1640 for 45 min, and placed on ice, centrifuged, resuspended in 100 μl ice-cold SDS sample buffer containing 100 mM dithiothreitol, and cells were lysed by boiling for 5 min. The cell lysates were separated by SDS-PAGE electrophoresis using 8–16% Tris-glycine polyacrylamide gradient gels. Separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P), and the membranes were subjected to immunoblotting with phospho-specific antibodies that recognize the phosphorylated forms of p38 MAPK (Thr180/Tyr182) and FAK (Tyr397 or Tyr576/577). As controls, membranes were also probed with antibodies that recognize both the active and inactive forms of p38 MAPK or FAK; i.e., using pan-p38 MAPK and pan-FAK antibodies. Bands were detected using X-ray films and LumiGLO chemiluminescent reagent (Cell Signaling Technology). Band intensities were analyzed with commercially available graphic software developed by the University of Texas Health Science Center in San Antonio (Image Tool 3.00), and ratios between band intensities of activated versus total p38 MAPK and FAK were used to calculate p38 MAPK and FAK activation rates.

Statistical analyses. Data are presented as means ± SD unless otherwise indicated. Differences between groups were evaluated by Student's *t*-test or analysis of variance (ANOVA) as indicated. Differences were considered significant at $P < 0.05$.

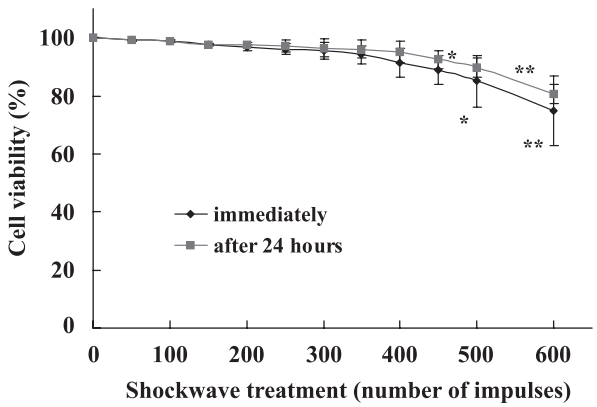

Fig. 2. Effect of shockwave treatment on Jurkat T-cell viability. After treatment with shockwaves with the indicated impulse numbers, cell viability was monitored with trypan blue exclusion. Viability was assessed within 5 min after shockwave treatment or after a 24-h culture period. Data are averages ± SD, $n = 6$, $*P < 0.05$, $**P < 0.01$; Student's *t*-test.

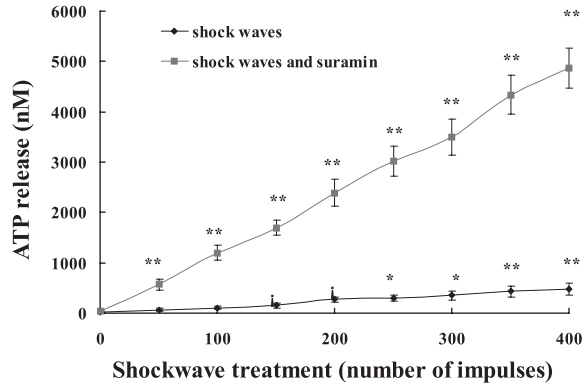

Fig. 3. Shockwaves cause rapid ATP release. ATP release into the culture supernatant from Jurkat T-cells (5×10^6/ml) was determined with a commercially available ATP Bioluminescence Assay Kit. Cells were allowed to rest for 3 h at 37°C before they were stimulated with shockwaves at 0.18 mJ/mm^2 with the indicated numbers of impulses in the absence or presence of suramin at a concentration of 100 μM, which prevents ATP degradation. Data are representative of 3 different experiments and expressed as means ± SD. Asterisks indicate statistically significant differences from control values (relative to no shockwave treatment) in the presence or absence of suramin. Statistics: $^{i}P < 0.05$, $*P = 0.01$, $**P < 0.001$; Student's *t*-test.

RESULTS

Effect of LDSW and different drug treatments on cell viability. ATP is released from intact cells subjected to membrane stretch. However, damaged cells also released ATP. Therefore, we investigated how shockwaves and the different drugs used in this study affect cell viability. Viability of shockwave-treated cells was assayed immediately (i.e., within 5 min after treatment) or after a 24-h culture period (Fig. 2). Viability of cells subjected to <300 LDSW impulses remained at >95% of untreated controls. Cells exposed to >500 impulses showed significantly decreased viability ($n = 6$, $*P < 0.05$, $**P < 0.01$). Treatment of cells with the different agents used to study shockwave-induced signaling only marginal affected cell viability (data not shown). Treatment with FAK siRNA, nontargeting control siRNA, 20 U/ml apyrase, 0.2 μM KN-62, 100 μM ATP, or 100 μM suramin had no significant ($P > 0.05$) effects and cell viability.

Shockwave treatment causes ATP release from Jurkat cells. The data above indicate that shockwave treatment with increasing impulse numbers gradually reduces cell viability by ~10% at 350 impulses (Fig. 2). This suggests that cells damaged by shockwave treatment may release ATP into the extracellular environment. Next we investigated ATP release in response to shockwaves. LDSW exposure caused a considerable and dose-dependent increase in extracellular ATP concentrations (Fig. 3). In the absence of suramin, ATP concentrations rose from a baseline level of 20 to 480 nM. ATP can be rapidly hydrolyzed by ecto-apyrases, ecto-ATPases, and ecto-5′-nucleotidases expressed on the cell surface of Jurkat cells (10). When suramin was added at a concentration known to block these enzymes and the breakdown of released ATP (24, 41), we found ~10 times higher ATP concentrations after shockwave treatment (Fig. 3). Thus our findings indicate that ATP is released into the extracellular space where it is partially hydrolyzed by enzymes expressed by Jurkat cells.

Shockwave treatment and extracellular ATP activate FAK in Jurkat T-cells. We then studied whether shockwave treatment affects FAK phosphorylation. LDSW treatment caused a considerable and dose-dependent increase in the phosphorylation of the FAK at Tyr residues 397 and 576/577, which is a reflection of FAK activation (Fig. 4). FAK activation increased with increasing numbers of impulses applied and it culminated at 250 impulses (Fig. 4). FAK activation decreased in cells treated with higher numbers of impulses, reaching baseline levels at 350 impulses (Fig. 4). To determine whether ATP released from cells could be responsible for FAK activation, we studied how exogenously added ATP affects FAK phosphorylation in Jurkat T-cells. Extracellular ATP added at concentrations $\leq$0.01 μM caused a dose-dependent increase in the phosphorylation of FAK on residues Tyr397 and 576/577, peaking at 1 μM ATP and gradually diminishing at higher ATP concentrations (Fig. 5). Taken together these findings suggest that ATP released in response to shockwave treatment induces FAK activation in a dose-dependent fashion.

Removal of ATP and inhibition of P2 receptors prevents shockwave-induced FAK phosphorylation. Loomis et al. (24) and Yip et al. (45) have shown that extracellular ATP influences Jurkat cell activation through P2-type purinergic receptors, possibly of the P2X7 receptor subtype. Therefore, we investigated whether shockwave-induced ATP release modulates FAK activation via P2X7 receptors. Jurkat cells were pretreated with apyrase, an enzyme that hydrolyzes extracellular ATP (24) or with KN-62, a purinergic receptor antagonist with specificity for the P2X7 receptor subtype (24, 28). The cells were then subjected to LDSW treatment by using 250 impulses, and FAK activation was determined by measuring tyrosine phosphorylation. Addition of apyrase or KN-62 caused a dose-dependent and significant reduction of FAK phosphorylation, suggesting that P2X7 receptors and extracellular ATP play important roles in the activation of FAK in response to shockwave treatment (Fig. 6). Although KN-62 also inhibits CaM Kinase II, about 10–100 times higher concentrations are needed compared with the KN-62 concentrations used in our study (3, 39).

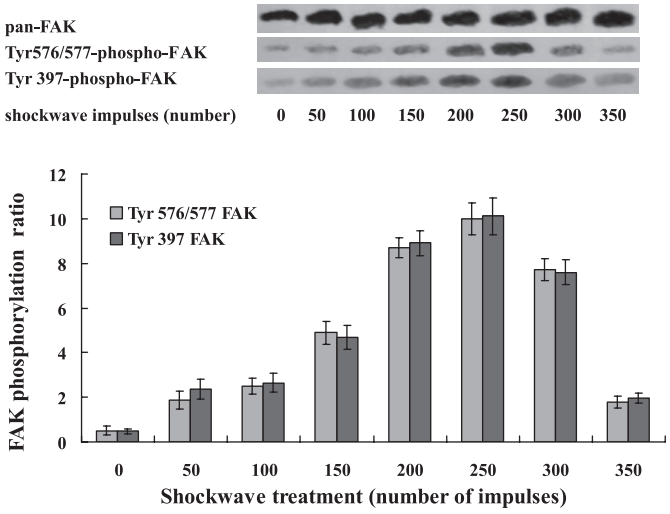

Fig. 4. Shockwave treatment activates focal adhesion kinase (FAK) in Jurkat T-cells. After shockwave treatment at 0.18 mJ/mm^2 with 0 (control) or the indicated numbers of impulses, Jurkat T-cells (10^6/ml) were cultured in 1 ml RPMI-1640 medium containing 10% FBS in a 12-well plate for 3 min, and FAK activation was determined by immunoblotting with antibodies recognizing FAK phosphorylated on residues Tyr397 or Tyr576/577 or antibodies recognizing the activated and inactive forms of FAK. Band intensities were analyzed and ratios between activated and total FAK were used to calculate FAK Tyr397 or 576/577 activation. Representative Western blots of 6 different experiments are shown and data were averaged in the bar graph (n = 6, means ± SD).

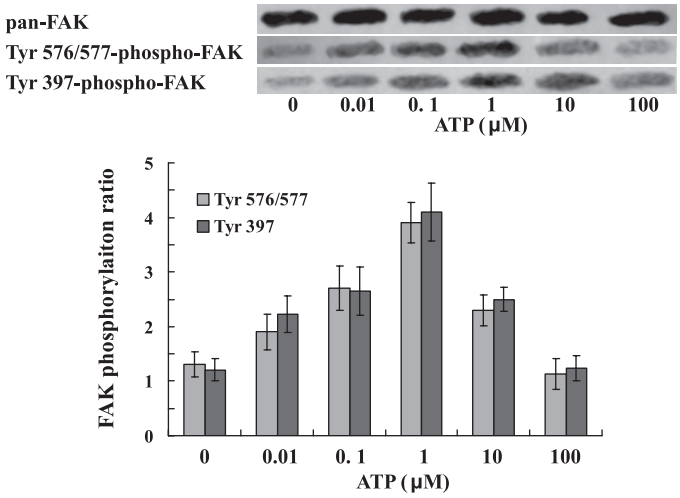

Fig. 5. Extracellular ATP activates FAK in Jurkat T-cells. Jurkat T-cells (10^6/ml) were cultured in 1 ml RPMI-1640 containing 10% FBS in a 12-well plate in the absence or presence of ATP for 3 min, and FAK activation was determined as described in Fig. 4. Representative Western blots of 6 different experiments are shown and data were averaged in the bar graph (n = 6, means ± SD).

FAK activation induces p38 MAPK phosphorylation. Previous work has shown that extracellular ATP enhances T-cell proliferation by upregulating IL-2 expression in a p38 MAPK-dependent fashion (24, 47). Here we found that shockwave-induced ATP release activates FAK. Next, we investigated whether FAK activation is required for downstream activation of p38 MAPK. FAK expression of Jurkat cells was downregulated using siRNA constructs that target FAK expression.

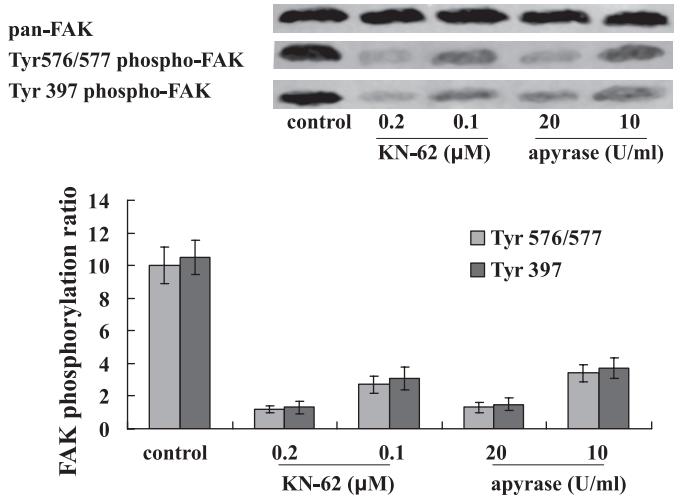

Fig. 6. Apyrase or KN-62 inhibits shockwave-induced FAK activation in Jurkat T-cells. Cells were subjected to shockwave treatment (0.18 mJ/mm^2 for 250 impulses) in the absence or presence of 10 U/ml apyrase, 20 U/ml apyrase, 0.1 μM KN-62, or 0.2 μM KN-62, and FAK activation was determined as described in Fig. 4. Representative Western blots of 6 different experiments are shown and data were averaged in the bar graph (n = 6, means ± SD).

Silencing FAK reduced the expression of FAK by 70% compared with control cells treated with nontargeting control siRNA constructs. FAK silencing blocked downstream p38 MAPK phosphorylation (Fig. 7*A*). Inhibition of P2X7 with KN-62 or elimination of release ATP with apyrase dose-dependently reduced p38 MAPK phosphorylation (Fig. 7*B*), suggesting that extracellular ATP, P2X7 receptors, and FAK activation contribute to shockwave-induced p38 MAPK phosphorylation.

Shockwaves enhance T-cell proliferation and IL-2 expression via the release of ATP, P2X7 receptors, and FAK activation. The data above indicate that ATP, P2X7 receptors, and FAK increase p38 MAPK activation in response to shockwaves. Next, we tested whether these events lead to increased IL-2 expression and T-cell proliferation. IL-2 expression and T-cell proliferation were induced by anti-CD3/CD28 stimulation. We found that silencing FAK with siRNA, removing released ATP with apyrase, or inhibiting P2X7 receptors with KN-62 completely abrogated shockwave-induced enhancements of T-cell proliferation and IL-2 expression (Figs. 8 and 9). These data show that FAK activation, release of ATP, and feedback through P2X7 receptors upregulate T-cell function in response to shockwave treatment.

DISCUSSION

We have previously reported that LDSW treatment of Jurkat cells enhances T-cell proliferation and IL-2 expression by activating p38 MAPK (47). In the present study, we report that these events are mediated by the release of ATP and autocrine feedback mechanisms that involve P2X7 receptors as well as FAK activation. The activation of FAK and p38 MAPK was decreased by apyrase, suramin, and the P2X7-specific antagonist KN-62. Silencing FAK, so as to decrease the expression of FAK, significantly reduced phosphorylation of p38 MAPK in response to shockwave treatment. This suggests that ATP, stimulation of P2X7 receptors, and activation of FAK are events that occur upstream of p38 MAPK and that these events in turn induce IL-2 expression and T-cell proliferation.

P2X7 receptors are abundantly expressed on the surface of Jurkat T-cells (45). These receptors belong to the seven different members of the P2X subfamily of P2 nucleotide receptors

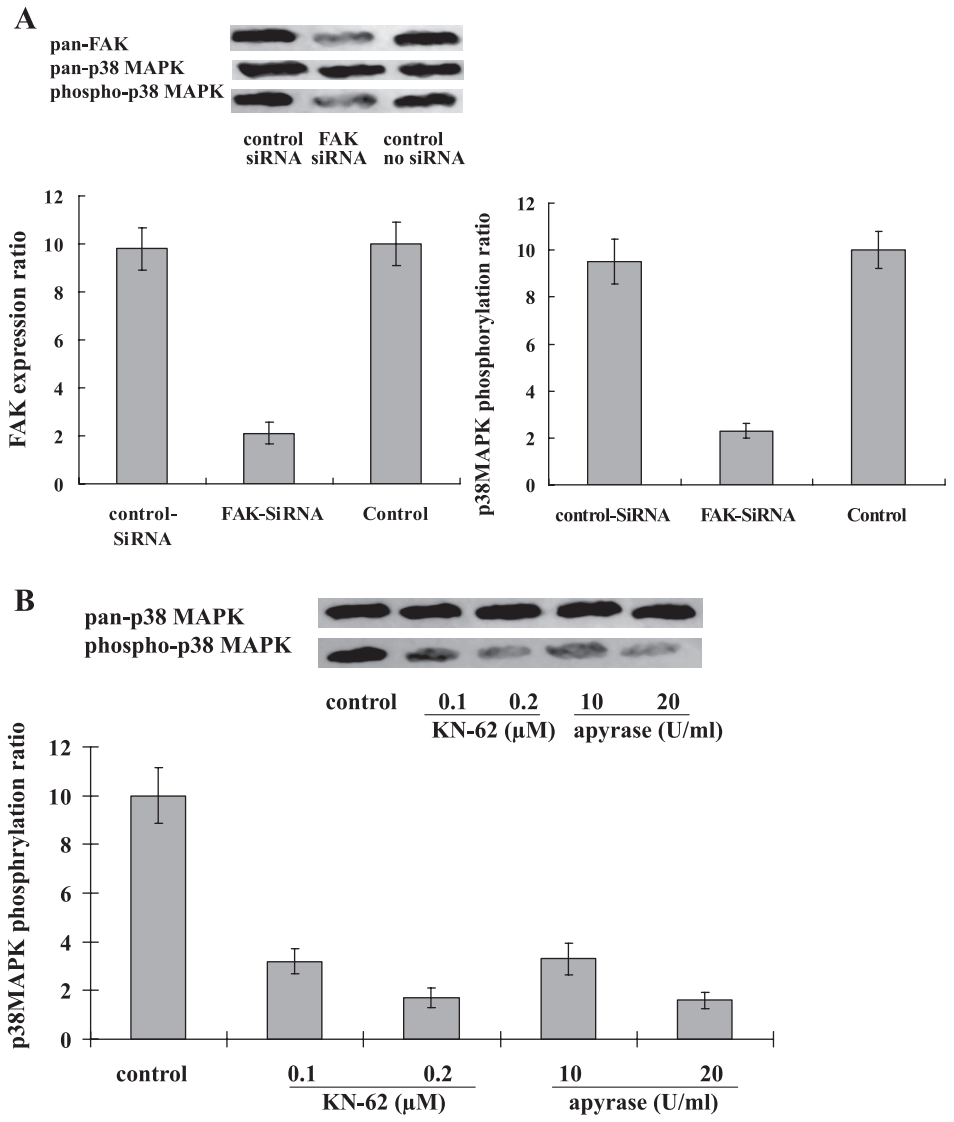

Fig. 7. Silencing of FAK and inhibition of purinergic signaling inhibits shockwave-induced p38 MAPK activation in Jurkat T-cells. Cells were treated with small interfering RNA (siRNA) constructs targeting FAK or with nontargeting siRNA construct (control siRNA), or with transfection agent without siRNA (control, no siRNA) (*A*) or with the P2X7 receptor antagonist KN-62 or with apyrase (*B*). After silencing or treatment with these agents, cells were subjected to shockwave treatment (0.18 mJ/mm^2 for 250 impulses), and FAK, p38 MAPK and activated p38 MAPK were measured as described in Fig. 6. Representative Western blots of 6 different experiments are shown and data were averaged in the bar graphs ($n = 6$, means ± SD).

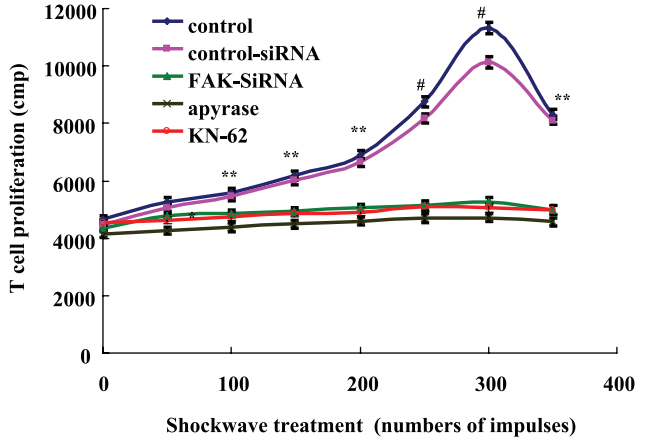

Fig. 8. Silencing of FAK or treatment with apyrase or KN-62 prevents the enhancing effect of shockwaves on phytohemagglutinin (PHA)-stimulated T-cell proliferation. Isolated human peripheral blood mononuclear cells (PBMCs) were treated as described, and T-cell proliferation was determined. Data were obtained from 3 different experiments and values are expressed as means ± SD. Asterisks indicate statistically significant differences compared with control values (no shockwave treatment) in the absence of FAK-siRNA, apyrase, or KN-62; ANOVA, $*P < 0.05$, $**P < 0.01$, $\#P < 0.001$.

(19). P2X receptors function as ATP-gated, nonselective ion channels that are permeable to Na^+, K^+, and Ca^{2+} (18). P2X receptors play important roles in T-cell activation by facilitating influx of extracellular calcium (45). FAK is a nonreceptor tyrosine kinase that resides at sites of integrin clustering, known as focal adhesions. FAK tyrosine phosphorylation is associated with the activation of receptor-coupled G proteins (52). The other family of purinergic receptors, the P2Y-type receptors, are G protein coupled and they are also expressed in T-cells (45). Hence, it is possible that these receptors may also contribute to shockwave-induced FAK and p38 MAPK activation.

MAPKs play vital roles in many cell functions including proliferation and cell differentiation (30). As a subfamily of the MAPK family, p38 MAPK is preferentially activated by distinctive stimuli such as UV irradiation, reactive oxygen species, and cellular or environmental stresses (30). Although G protein-coupled receptors are known to play a major role in the activation of p38 MAPK (2, 30, 42), our findings with receptor antagonists imply that P2X7 receptors are responsible for the activation of p38 MAPK in Jurkat cells subjected to shockwave treatment. P2X7 thus elicits secondary signaling responses that include calcium signaling and p38 MAPK activation.

Our data show that FAK activation is crucial for shockwave-induced phosphorylation of p38 MAPK. The activation state of FAK is defined largely by the phosphorylation of Tyr397, an autophosphorylation site of the molecule (9). Tyr397 phosphorylation creates a high-affinity binding site for the Src SH2 domain that is required for the recruitment and activation of Src (9). Formation of a complex with Src is arguably the most critical event in FAK-associated signaling (8, 9). Src bound to the Tyr397 site phosphorylates other FAK residues including residues Tyr576 and Tyr577 in the kinase domain activation loop. These sites are important for maximal FAK autophosphorylation activity (8, 9). Src family kinases play important roles in phosphorylation of MAPKs (27, 37). Thus our findings imply that shockwaves can induce tyrosine phosphorylation of FAK, which in turn recruits and activates Src, ultimately causing the phosphorylation (and thereby activation) of p38 MAPK.

When cells are in a steady state under normal physiological conditions, the level of ATP outside the cells is well balanced due to the rate of basal or constitutive ATP release (24) and the rate of ATP hydrolysis by ecto-ATPases and ecto-nucleotidases (27). This steady-state resting level of the extracellular ATP concentration seems to be necessary for normal physiological functions of T-cells because extracellular ATP binds and activates P2 receptors in autocrine and paracrine fashions. If the steady-state extracellular nucleotide balance is lost, normal immune cell function is altered, which can result in changed T-cell proliferation and differentiation, modified chemotactic responses of neutrophils, and altered patterns of cytokine expression and release of lysosomal constituents of a multitude of different cell types (27). We found that shockwave treatment increases extracellular ATP concentrations drastically, resulting in complex signaling responses that increases T-cell function. Depending on the frequency of the shockwave treatment, ATP release may be a result of mechanical cell deformation that induces the opening of specific release channels and liberation of ATP from damaged cells.

Shockwaves are used to target musculoskeletal disorders such as pseudoarthosis, tendinitis, calcarea of the shoulder, epicondylitis, plantar fasciitis, and several inflammatory tendon diseases (13, 23, 31, 36, 53). Shockwave treatment results in statistically significant improvements in clinical outcome with a reduction of pain and inflammation (13, 16, 23, 31, 36, 53). The cell types involved in the pathology of these conditions may also be susceptible to shockwave treatment via mechanisms similar to those elucidated in T-cells. Therefore, ATP release from these cell types and feedback through purinergic receptors could be the underlying principle by which shockwave treatment can improve the symptoms of these different diseases. Shockwaves induce long-term tissue regeneration effects that facilitate healing processes in treated tendons and muscle tissues (13, 31). Rapid enhancements of

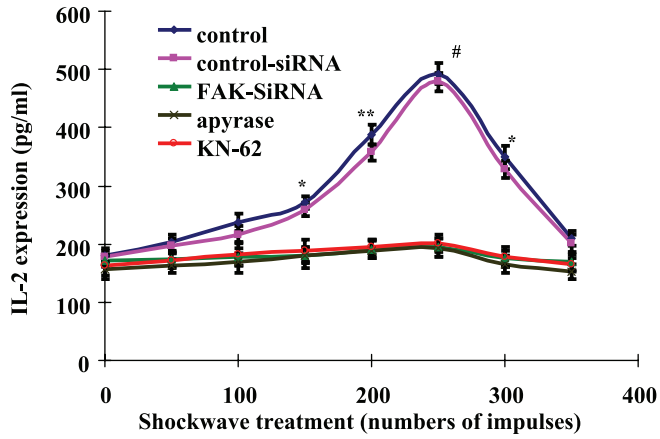

Fig. 9. Silencing of FAK or treatment with apyrase or KN-62 prevents the enhancing effect of shockwaves on CD3/CD28 stimulated IL-2 expression. Isolated human PBMCs were treated as described, and IL-2 production was measured. Data are representative of 3 different experiments, and values were expressed as means ± SD. Asterisks indicate statistically significant differences from control values (no shockwave treatment) in the absence of FAK-siRNA, apyrase, or KN-62; ANOVA $*P < 0.05$, $**P < 0.01$, $\#P < 0.001$.

endothelial nitric oxide synthase (eNOS) activity in shockwave-treated cells and subsequent suppression of nuclear factor (NF)-κB activation may be potential additional mechanisms by which shockwave treatment elicits its beneficial action in the treatment of tissue inflammation (1, 4, 22).

Our work has shown that LDSW treatment may be useful to modulate immune function. However, this possibility has not yet been tested clinically or in animal models. Such additional studies are needed to examine the possibility of utilizing shockwave treatment to modulate immune responses in vivo. In addition, the dose and mode of administration of shockwave treatment will have to be defined to optimize its potential effectiveness as a regulator of immune function and to also investigate possible negative side effects that may result from shockwave treatment.

Our findings suggest that shockwaves cause cell damage, which may provide the ATP that activates P2X7 receptors and the downstream responses that influence T-cell functions. Since shockwaves have been shown to cause cell permeabilization, which facilitates the entry of macromolecules and small polar molecules into the cytoplasm (20, 21, 40), intracellular molecules including ATP may be released in turn from such permeabilized cells. However, it is possible that mechanical stress associated with shockwave treatment may trigger ATP release through additional mechanisms (33). Possible candidates would be stretch-activated pores and channels including gap junction hemichannels such as connexin-43 and pannexin-1 that have been recently shown to facilitate ATP release from leukocytes (11, 32). In our future work, we plan to investigate which of these mechanisms are involved in the release of ATP from shockwave-treated T-cells.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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