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ATP Induces Protein Arginine Deiminase 2-Dependent Citrullination in Mast Cells through the P2X7 Purinergic Receptor

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Posttranslational modifications regulate physiology either by directly modulating protein function or by impacting immune recognition of self-proteins. Citrullination is a posttranslational modification formed by the conversion of arginine residues into the citrulline amino acid by protein arginine deiminase (PAD) family members. We have identified mast cells as a major source of the PAD2 enzyme. Activation of the P2X7 purinergic receptor (P2X7) by the inflammatory “danger” signal ATP induces PAD2 activity and robust protein citrullination. P2X7-mediated activation of PAD2 is sensitive to p38 MAPK and protein kinase C inhibitors, and PAD2 regulates the expression of the TNFR2, Adams-9, and Rab6b transcripts in mast cells. Further, the PAD2 enzyme and its citrullinated substrate proteins are released from mast cells on activation with ATP. PAD2 expression is closely linked with inflammation in rheumatoid arthritis (RA) synovial tissue, and PAD2 and citrullinated proteins are found in the synovial fluid of RA patients. In addition, RA is associated with the development of autoantibodies to citrullinated self-proteins. Our results suggest that P2X7 activation of mast cells may play a role in inflammation by providing PAD2 and PAD2 substrates access to the extracellular space. The Journal of Immunology, 2012, 189: 4112–4122.

Citrulline-containing proteins are generated through posttranslational modification of arginine residues in a reaction catalyzed by the Ca2+-dependent peptidyl arginine deiminases. The conversion of arginine to citrulline results in a small change in molecular mass (<1 Da) and in a loss of a positive charge, which can have dramatic consequences on protein structure and protein–protein interactions. There are five mammalian protein arginine deiminase (PAD) family members, PAD1–4 and PAD6 (1). In the immune system, PAD2 and PAD4 are the most likely candidates to regulate inflammation because they are both expressed in hematopoietic cells, whereas the expression of PAD1, PAD3, and PAD6 is restricted to the epidermis, hair follicle, and oocyte, respectively (1). PAD2 is a ubiquitously expressed member of the PAD2 family, with high level of expression documented in the CNS, monocytes, macrophages, and keratinocytes (1). Keratins, filaggrin, vimentin, myelin basic protein, fibrinogen, chemokines, and histones are all known PAD substrates (2). Treatment with the Ca2+ ionophore ionomycin induces endogenous PAD activity and subsequent protein citrullination (1). However, the possibility of PAD activation through regulation of the enzymes by factors other than calcium has not been explored in detail. In fact, little is known about the link between physiological signals and the intracellular Ca2+ increase that leads to PAD-mediated modifications.

Under steady-state conditions, extracellular ATP levels are low; however, under inflammatory conditions, activated and dying cells, degranulating platelets, and pathogenic bacteria release high concentrations of the “danger-signal” ATP into the extracellular space, stimulating the innate immune response (3, 4). P2X7 purinergic receptor (P2X7) is an ATP-gated cation channel, predominantly expressed on immune cells, that requires high levels of ATP for activation (5). Brief stimulation of the P2X7 induces Ca2+ flux and downstream receptor signaling (6). P2X7 stimulation activates several signaling pathways, including protein kinase C (PKC), MAPK pathways, NFAT, and NF-κB pathways (7). Ultimately, P2X7 activation leads to the production of inflammatory molecules such as IL-6 and TNF-α (6). ATP-induced stimulation of P2X7 also triggers the NALP3 inflammasome, leading to the activation of caspase-1 and the processing and release of IL-1β (8). Although bursts of ATP exposure can lead to cell proliferation, prolonged P2X7 stimulation leads to the formation of a large pore that permits the passage of hydrophilic molecules as large as 900 Da and depolarization of membrane potential, which can lead to cell death in some cell types (9, 10). The induction of multiple inflammatory effectors downstream of P2X7 makes ATP a relevant physiological stimulus in inflammation and autoimmunity.

Rheumatoid arthritis (RA) is a frequent and chronic inflammatory disease of the synovial joints. Plasma and synovial biopsy specimens from patients with RA contain high levels of citrullinated proteins, and anti-citrullinated peptide Abs (ACPA) exhibit high specificity and sensitivity as diagnostic markers of the disease (11). PAD2 is highly expressed in synovial tissue of RA patients, in close association with citrullinated protein deposits, and its expression correlates with inflammation intensity (12, 13). Most PAD2-expressing cells within the RA synovium are positive for CD68, a marker of macrophages, dendritic cells, neutrophils, and mast cells (12). In an effort to determine the cellular source of synovial PAD2 and citrullinated proteins, we identified mast cells as a major PAD2-expressing cell type. Upon stimulation, mast cells
cells rapidly release mediators prestored in their granules, such as histamine, heparin, and a variety of proteases; synthesize and secrete cytokines and lipid mediators; and contribute to the recruitment of the cells of the innate and adaptive immune system (14). We report in this article that activation of PAD2 by ATP is a novel signaling pathway in mast cells that leads to the activation of PAD2 and the release of PAD2 and citrullinated proteins into the extracellular space.

Materials and Methods

Animals

PAD2−/− mice were obtained from Lexicon Genetics (Woodlands, TX) and backcrossed onto C57BL/6J background for 12 generations to obtain C57BL/6-Pad2−/− mice (PAD2−/− mice). PAD2−/− mice were obtained from The Jackson Laboratory (15). C57BL/6J and C57BL/6-Pad2−/− mice were bred at The Scripps Research Institute and housed according to The Scripps Research Institute Institutional Animal Care and Use guidelines.

Abs and reagents

Mouse sera specific for PAD2 were obtained by immunizing PAD2−/− mice with purified recombinant GST-tagged murine PAD2 fusion protein. Rabbit PAD2-specific Ab was from ProteinTech Group (Chicago, IL). Abs specific for actin and tubulin were from Abcam (Cambridge, MA). Abs specific for p38 MAPK and phosphorylated p38 MAPK were from Cell Signaling Technology (Danvers, MA). PE-conjugated anti-FcεRI Ab, FITC-conjugated Annexin V, unconjugated and biotin-conjugated mouse-specific anti-IL-6 Abs were from ebioscience (San Diego, CA). Unconjugated and biotin-conjugated Abs specific for mouse TNF-α and IL-13 were from R&D Systems (Minneapolis, MN). PE-Cy7-conjugated mouse-specific Ab for c-Kit (CD117), TNF-specific IgG1 (C38-2), and rat anti-mouse CD16/CD36 (2.4G2) were from BD Biosciences (San Jose, CA). TNP-BSA was from Biosearch Technologies (Novato, CA). Goat F(ab')2 anti-rat IgG was from Southern Biotech (Birmingham, AL). Propidium iodide (PI) was from MP Biomedicals (Solon, OH). Alkaline phosphatase-conjugated avidin, phosphatase substrate tablets, 2-ME, sodium orthovanadate, and sodium deoxycholate were from Sigma (St. Louis, MO). PMA, ionomycin, L9294002, PD98059; INK inhibitor II, p38 MAPK inhibitor III, bisindolylmaleimide, and necrostatin-1 were from EMD (Gibbstown, NJ). Indo-1 AM was from Invitrogen (Carlsbad, CA). HEPES was from The Jackson Laboratory (15). C57BL/6J and C57BL/6- (Pittsburgh, PA). HEPES was obtained by incubating PAD2+ mice with Tyrodes buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHPO4, 0.1 mM MgCl2, 1.3 mM CaCl2, 0.1% gelatin, and 10 mM HEPES, pH 7.3), and 5 × 105 cells were plated in 100 µl/well of a 96-well plate. BMNC degranulation was induced by 1 mM ATP or with 50 ng/ml PMA and 1 µM ionomycin. Unstimulated cells were used as controls. After 1 h at 37°C, the cells were pelleted by centrifugation, and cell extracts were prepared in PBS containing 1% Triton X-100. The amount of mast cell enzyme β-hexosaminidase activity in cell supernatants and extracts was measured using the substrate 4-nitrophenyl-N-acetyl-β-D-glucosaminide. Degranulation was plotted as percentage of β-hexosaminidase activity detected in the cell supernatants compared with total activity present in the supernatants and cell extracts.

BMMC supernatant collection

Supernatants were collected; protease inhibitors, PMSF, and sodium orthovanadate were added as described above; and the supernatants were concentrated using the Amicon Ultra concentrators with the 10,000 m. w. cutoff limit (Millipore). Pelleted cells were washed once with cold PBS, and cell extracts were prepared as described above.

Cytokine ELISA

BMMC were stimulated with 1 mM ATP for 24 h at 37°C. Unstimulated cells were used as controls. Cell supernatants were used in a sandwich ELISA assay to determine the level of IL-6, IL-13, and TNF-α, following manufacturer’s instructions.

BMMC degranulation assay

Degranulation assays were performed as previously described (16). In brief, BMMC were washed once with Tyrodes buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHPO4, 0.1 mM MgCl2, 1.3 mM CaCl2, 0.1% gelatin, and 10 mM HEPES, pH 7.3), and 5 × 105 cells were plated in 100 µl/well of a 96-well plate. BMNC degranulation was induced by 1 mM ATP or with 50 ng/ml PMA and 1 µM ionomycin. Unstimulated cells were used as controls. After 1 h at 37°C, the cells were pelleted by centrifugation, and cell extracts were prepared in PBS containing 1% Triton X-100. The amount of cell enzyme β-hexosaminidase activity in cell supernatants and extracts was measured using the substrate 4-nitrophenyl-N-acetyl-β-D-glucosaminide. Degranulation was plotted as percentage of β-hexosaminidase activity detected in the cell supernatants compared with total activity present in the supernatants and cell extracts.

BMMC Ca2+-flux

BMMC were sensitized with IgE for Ag stimulation or vehicle for ATP stimulation for 16 h at 37°C. The cells were then washed and loaded with 0.25 µM Indo-1 AM for 30 min at 37°C. BMMC were washed twice with complete media and once with Ca2+-free media (containing HBSS supplemented with 10% FBS, 1 mM HEPES, 1 mM MgCl2, and 1 mM EGTA) and resuspended at 2 × 106 cells/ml in Ca2+-free media. Changes in violet and blue Indo-1 fluorescence with time were determined by flow cytometry after the addition of 50 ng/ml TNP-BSA or 1 mM ATP, 10 mM CaCl2 and 1 µg/ml ionomycin at the different time points. The violet/blue ratio was plotted over time as relative Ca2+-flux.

Statistics

Average results are expressed as the mean ± SEM. Data were analyzed by an unpaired Student’s t test (GraphPad Prism software) to determine statistical significance.

Results

PAD2 is expressed in mast cells

Because PAD2 is found within the synovial fluid of RA patients and correlates with citrullinated protein deposits (12, 13), we sought to identify potential cellular sources and signaling pathways that...
would lead to extracellular-residing PAD2. We first examined the PAD2 expression profile compared with other tissues and cell types, particularly those associated with the immune system. We prepared RNA from murine tissues and performed qPCR to assess PAD2 mRNA expression. PAD2 was reported to be ubiquitously expressed (1). Indeed, we found that PAD2 is widely expressed: we detected PAD2 mRNA in lymph node, thymus, spleen, heart, kidney, skeletal muscle, brain, liver, and bone marrow samples (Fig. 1A). As has been previously reported, PAD2 expression was particularly enriched in skeletal muscle (17). Because mast cells accumulate in human rheumatoid synovial tissues (18), we established primary cultures of BMMC to examine whether PAD2 is expressed in this cell type. We discovered very high levels of PAD2 expression in BMMC, paralleling those observed in the skeletal muscle (Fig. 1A). We also readily detected the PAD2 protein in BMMC (Fig. 1B). To ensure that our Ab was not cross-reacting with other PAD family members, we made use of BMMC derived from PAD2−/− mice (19) (Fig. 1B). Importantly, maturation of BMMC from wild type (WT) and PAD2−/− cultures was comparable, as determined by FcεRI and c-Kit staining (Fig. 1C).

To determine PAD family expression profile in mast cells, we prepared RNA from BMMC and performed qPCR analysis. As expected, the PAD2 transcript was expressed at robust levels (Fig. 1D). In contrast, PAD1 expression was not detected in BMMC (data not shown). In addition, PAD3 and PAD6 transcripts were nearly undetectable (Fig. 1D). Although the PAD4 transcript was detected at low levels (Fig. 1D), we were unable to detect protein expression of PAD4 in BMMC by immunoblot (data not shown). Importantly, deletion of PAD2 in BMMC did not result in compensatory up-regulation of other PAD transcripts, supporting the comparison between WT and PAD2−/− BMMC for these studies (Fig. 1D). Thus, PAD2 expression is prominent in murine mast cells.

**ATP activates PAD2-dependent protein citrullination in mast cells**

The presence of Ca2+ is essential for the enzymatic activity of PAD family members (20, 21). Structural studies of the PAD4 enzyme suggest that Ca2+ binding to residues conserved in PAD1–4 induces a conformational change, leading to the formation of the active site cleft (22). Because mast cells express high levels of PAD2 (Fig. 1) and stimulation through purinergic receptors can induce Ca2+ influx (23), we examined whether ATP could induce PAD activation in BMMC by monitoring protein citrullination. Incubation of BMMC with ATP leads to rapid and robust protein citrullination (Fig. 2A). In our hands, ATP stimulation of BMMC for 60 min does not induce cell death, as assayed by Annexin V and PI staining (Fig. 2B, 2C). Furthermore, inhibition of mast cell apoptosis with the caspase inhibitor Z-VAD-FMK (Fig. 2D) or cell necrosis with necrostatin-1 (Fig. 2E) did not inhibit ATP-induced protein citrullination. Taken together, these data suggest that ATP-induced protein citrullination is likely independent of cell death.

Next, we determined the extent to which ATP-induced mast cell protein citrullination was dependent on PAD2. We stimulated WT and PAD2−/− BMMC with ATP and examined citrullination by immunoblot. The ATP-induced mast cell citrullination, at least to the level of detection by immunoblot, was completely dependent on the presence of PAD2 (Fig. 3A). Mast cell protein citrullination in response to the Ca2+ ionophore ionomycin was also PAD2-dependent.

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**FIGURE 1.** PAD2 is expressed in mast cells. (A) Total RNA was extracted and cDNA prepared from mouse tissues listed. qPCR was performed as described in Materials and Methods to detect PAD2 expression. A representative of two independent experiments is shown. (B) Cell extracts were prepared from the BMMC and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for PAD2. Cell extracts were immunoblotted for actin as a measure of protein load. A representative of more than three independent experiments is shown. (C) BMMC were stained with Abs specific for c-Kit and FcεRI, and subjected to flow cytometry analysis. Percentage of stained cells is shown in a representative of three or more experiments. (D) Total RNA was extracted and cDNA prepared from the WT (dark gray bars) or the PAD2−/− (light gray bars) BMMC. qPCR was performed as described in Materials and Methods to detect PAD2, PAD3, PAD4, and PAD6 expression. A representative of two independent experiments is shown. BM, Bone marrow; LN, lymph node; SM, skeletal muscle.
dependent (Fig. 3A). In contrast, PAD4 activity was dispensable because ATP-stimulated citrullination remained intact in BMMC derived from PAD4f1/fl CMV-Cre deleter mice (PAD4−/−; Fig. 3B) (24). These data suggest that PAD2 plays the predominant role in ATP-induced citrullination within the mast cell compartment.

Regulated Ca2+ mobilization is a feature of many mast cell receptor activation pathways, such as FcεRI and FcγRII/III signaling (25). To determine whether receptor-mediated Ca2+ mobilization was sufficient to induce PAD2, we incubated BMMC precoated with TNP-specific IgE with TNP-BSA to stimulate FcεRI, or we cross-linked the FcγRII/III with an mAb. In contrast with stimulation with ATP, neither FcεRI (Fig. 3D) nor FcγRII/III (Fig. 3E) induced PAD2 activity. Thus, the activation of PAD2 and its subsequent protein citrullination is specific for ATP stimulation.

Mast cell ATP-induced citrullination requires the P2X7 receptor

ATP is a ligand for several purinergic receptors. To determine the purinergic receptor responsible for ATP-induced citrullination, we surveyed the expression profile of purinergic receptors expressed by mast cells by qPCR. As is shown in Fig. 4A, BMMC expressed transcripts for P2X receptors P2X1, P2X4, and P2X7. P2X1 was the only P2Y receptor expressed by BMMC (Fig. 4B). Importantly, P2 receptor expression levels were unaffected by the absence of PAD2 (Fig. 4A, 4B). The P2X1, P2X4, and P2X7 receptors all promote the influx of extracellular Ca2+ (23). Because we found the threshold for ATP-induced protein citrullination detection to be ≥250 μM (data not shown), the pharmacological evidence supported the notion that P2X7, which has low affinity for ATP and, therefore, requires high levels of ATP for activation, may be responsible for inducing ATP-stimulated, PAD2-dependent protein citrullination (23). Indeed, ATP stimulation of P2X7−/− BMMC failed to induce protein citrullination (Fig. 4C). In addition, pharmacological interference with P2X7 function by compound A438079 also blocked citrullination (Fig. 4D). However, P2X7−/− BMMC are not simply incapable of activating PAD2 because PMA and ionomycin stimulation induced protein citrullination in P2X7−/− BMMC (Fig. 4E). These data support the notion that ATP induces PAD2 activity by signaling through the P2X7.

PAD2 activation by ATP requires p38 MAPK and PKC activity

Ligation of P2X7 by ATP induces several second messenger and enzymatic cascades (7, 26). We investigated the position of PAD2 within P2X7 signaling cascades. Because P2X7 is an ATP-gated cation channel that induces Ca2+ flux, and the PAD proteins require high levels of Ca2+ to be activated, we tested the importance of extracellular Ca2+ in the activation of PAD2 by ATP (5, 20, 21). In fact, we found that ATP-induced PAD2 activation requires
FIGURE 3. ATP-induced citrullination in BMMC requires PAD2. (A) BMMC from the WT or PAD2−/− mice were stimulated with ATP (1 mM), 50 ng/ml PMA and 1 μM ionomycin (PMA/I), or vehicle (control) for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for tubulin as a measure of protein load. A representative of three or more independent experiments is shown. (B) BMMC from the WT or PAD4−/− mice were stimulated with ATP (1 mM) or vehicle for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for p38 MAPK as a measure of protein load. A representative of two independent experiments is shown. (C) BMMC were sensitized with IgE for Ag stimulation or vehicle for ATP stimulation for 16 h at 37°C. The cells were then washed, loaded with Indo-1–AM, and kept in Ca2+-free medium. Intracellular Ca2+ levels over time were measured as the following stimuli were added at the time points indicated by the arrows: Ag (black trace), 1 mM ATP (gray trace), 10 mM CaCl2 (Ca2+), and 1 μg/ml ionomycin. A representative of two independent experiments is shown. (D) BMMC from the WT or PAD2−/− mice were stimulated with ATP (1 mM), 50 ng/ml PMA and 1 μM ionomycin (PMA/I), or vehicle (control) for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for tubulin as a measure of protein load. A representative of three or more independent experiments is shown. (Figure legend continues)
extracellular Ca\(^{2+}\) (Fig. 5A). To determine whether PAD2 is involved in ATP-induced Ca\(^{2+}\) mobilization, we loaded WT or PAD2\(^{2-/-}\) BMMC with Ca\(^{2+}\)-sensitive dyes, stimulated the cells with ATP, and measured the Ca\(^{2+}\) flux by flow cytometry. BMMC from WT and PAD2\(^{2-/-}\) mice exhibited essentially equivalent ATP-induced Ca\(^{2+}\) flux (Fig. 5B). Although the P2X7 signaling pathway has not been completely mapped, P2X7 stimulation is known to induce the activity of several kinases, including phospholipase D, PI3K, PKC\(\alpha\), PKC\(\beta\), PKC\(\gamma\), MEK/Erk1/2, JNK, and p38 MAPK (7, 27). In an effort to identify the P2X7 signaling pathways that converge on PAD2, we made use of several commercially available kinase inhibitors to assess their impact on PAD2-dependent protein citrullination. Before ATP stimulation, we treated cells with DMSO or the following inhibitors: 10 \(\mu\)M LY294002 (PI3K inhibitor), 50 \(\mu\)M PD98058 (MEK1/2 inhibitor), 50 nM Jnk II inhibitor, 50 nM p38 MAPK inhibitor III, 100 nM bisindolylmaleimide (PKC inhibitor), or 100 \(\mu\)M Rottlerin (PKC inhibitor). PAD2 activity was not affected by compounds that interfere with PI3K, MEK/Erk1/2, or JNK enzymes (Fig. 5C). However, incubation with p38 MAPK inhibitor III or Rottlerin before ATP stimulation largely prevented protein citrullination, whereas treatment with bisindolylmaleimide reduced citrullinated protein levels (Fig. 5C). Bisindolylmaleimide is selective for PKC\(\alpha\), \(\beta\), \(\beta\), \(\gamma\), \(\delta\), and \(\epsilon\) isozymes. At the higher levels, such as those used in this study, Rottlerin inhibits the PKC\(\alpha\), \(\beta\), \(\gamma\), \(\delta\), \(\epsilon\), \(\eta\), and \(\zeta\) isozymes, as well as CaM kinase III. The greater impact of Rottlerin on protein citrullination levels (Fig. 5C) may reflect its broader inhibitory profile. ATP-induced p38 MAPK activation

PAD2\(^{2-/-}\) mice were stimulated with ATP (1 mM, left panel), with IgE and Ag to activate the FceRI (right panel), or vehicle as described in Materials and Methods. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for tubulin as a measure of protein load. A representative of three independent experiments is shown. (E) BMMC from the WT or PAD2\(^{2-/-}\) mice were stimulated with 50 ng/ml PMA and 1 \(\mu\)M ionomycin (PMA/I), ATP (1 mM), or vehicle (Control) for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for actin as a measure of protein load. A representative of two independent experiments is shown.

FIGURE 4. ATP-induced citrullination in BMMC requires the P2X7. Total RNA was extracted and cDNA prepared from the WT (dark gray bars) or the PAD2\(^{2-/-}\) (light gray bars) BMMC. qPCR was performed as described in Materials and Methods to detect the P2X receptors (A), P2Y receptors (B), or PAD2 expression (A, B). A representative of two experiments is shown. (C) BMMC from the WT, PAD2\(^{2-/-}\), or P2X7\(^{2-/-}\) mice were stimulated with ATP (1 mM) or vehicle (control) for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for tubulin as a measure of protein load. A representative of three or more experiments is shown. (D) WT BMMC were pretreated with A438079 or vehicle control for 1 h before stimulation with ATP (1 mM) for 10 min. Cell extracts were analyzed as in (C). A representative of three independent experiments is shown. (E) BMMC from the P2X7\(^{2-/-}\) mice were stimulated with 50 ng/ml PMA and 1 \(\mu\)M ionomycin (PMA/I), ATP (1 mM), or vehicle (Control) for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for actin as a measure of protein load. A representative of two independent experiments is shown.
remained intact in PAD2−/− BMMC, whereas ATP-induced p38 MAPK activation was impaired in P2X7−/− BMMC (Fig. 5D). These results place ATP-induced PAD2 activation downstream of Ca2+ mobilization and p38 MAPK and PKC enzymatic activity.

**BMMC degranulation and cytokine secretion are not altered by PAD2 deficiency**

Stimulation of mast cells with a variety of agonists leads to the release of preformed mediators stored in granules, such as histamine, and to the synthesis of new inflammatory mediators, such as cytokines and chemokines (14). ATP is known to stimulate mast cell degranulation (28, 29). To examine the impact of PAD2 on ATP-induced mast cell degranulation, we stimulated the WT or PAD2−/− BMMC with ATP and monitored activity of Ca2+ mobilization and p38 MAPK and PKC enzymatic activity.

**PAD2 regulates gene expression in mast cells**

Citrullination results in the loss of positive charge with potentially dramatic consequences on protein intramolecular and intermolecular interactions, thereby impacting signal transduction networks and gene expression (1). Indeed, in addition to directly modifying chromatin, PAD4 regulates transcription by modifying the p300 GRIP1-binding domain of p300, which enhances the association between the p300 and GRIP1 transcriptional coactivators (31). Because signaling through P2X7 leads to gene expression changes in microglia (32, 33), we investigated the regulation of ATP-induced gene expression by PAD2 using a candidate gene approach. Mast cells express many TNF superfamily and TNF receptor family members (34). Molecules involved with TNF sensing are of particular interest because of their strong link to inflammation (35). In addition, polymorphisms within TNFR2 have been linked with RA (36). Interestingly, we found that ATP stimulation leads to upregulation of TNFR2 expression, which is diminished in PAD2−/− BMMC (Fig. 7A). Inflammatory stimuli, such as TNF-α and IL-1β, have been shown to induce expression of the Adamts-9 metalloproteinase (37). We found that ATP stimulation also induced Adamts-9 expression (Fig. 7B). There was a trend for ATP stimulation to upregulate Rab6b expression, which was absent in PAD2−/− BMMC (Fig. 7C). Importantly, activation of P2X7 triggers membrane trafficking events (40). We found that Rab6b is also expressed in BMMC (Fig. 7C). There was a trend for ATP stimulation to upregulate Rab6b expression, which was absent in PAD2−/− BMMC (Fig. 7C). In fact, baseline levels of Rab6b transcripts were lower in PAD2−/− BMMC. These data support the notion that PAD2 regulates both basal and ATP-induced mast cell gene expression.

**ATP induces release of PAD2 and citrullinated proteins**

PAD2 and citrullinated proteins are found within the synovial fluid of RA patients (41), but the mechanism by which these molecules

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**FIGURE 5.** ATP-induced citrullination in BMMC requires p38 MAPK and PKC activity. (A) WT BMMC were incubated in the absence (−) or presence (+) of 2 mM CaCl2 during the 10-min stimulation with ATP (1 mM). Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Ab specific for citrullinated proteins. Cell extracts were immunoblotted for tubulin as a loading control. A representative of three independent experiments is shown. (B) WT (black trace) and PAD2−/− (gray trace) BMMC were loaded with Indo-1–AM and kept in Ca2+-free medium. Intraplasmic Ca2+ levels over time were measured as the following stimuli were added at the indicated time points: 1 mM ATP, 10 mM CaCl2 (Ca2+), and 1 μg/ml ionomycin, indicated by the arrows. (C) BMMC were incubated with the following chemical inhibitors as described in Materials and Methods: LY294002 (LY), PD98059 (PD), JNK inhibitor II (JNK), p38 MAPK inhibitor III (p38), bisindolylmaleimide (Bis), Rottlerin (R), or vehicle (−) for 1 h before the 10-min stimulation with ATP (1 mM) or vehicle. Cell extracts were immunoblotted for actin as a measure of protein load. (D) BMMC from the WT, PAD2−/−, or P2X7−/− mice were stimulated with 1 mM ATP (+) or vehicle (−) for 10 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the Abs specific for PAD2 or phosphorylated p38 MAPK. Cell extracts were immunoblotted for p38 MAPK as a measure of protein load. Representatives of three independent experiments are shown.

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gain access to the extracellular space is unknown. Stimulation of P2X7 of macrophages triggers the release of large membrane complexes that contain macromolecules, such as IL-1β (42). Further, activation of “P2z” (the former designation of P2X7) by extracellular ATP was shown to induce the exocytosis of rat mast cell granules (43). Because stimulation of BMMC by ATP promotes degranulation (Fig. 6A) and PAD2-dependent protein citrullination (Fig. 3A), we hypothesized that activation of mast cells by ATP may lead to the release of citrullinated proteins. To test this possibility, we collected cell supernatants and lysates from WT or PAD2−/− mast cells that were either left untreated or were treated with ATP for 10 min and immunoblotted for protein citrullination. After stimulation of mast cells by ATP, we detected the presence of citrullinated proteins in the supernatants collected from WT but not from PAD2−/− BMMC (Fig. 8). Tubulin has been described as a resident of exosomes produced by B cells and by human mesothelial cells, and has been shown to be secreted by PMA-stimulated macrophages (44–46). Immunoblotting for tubulin provided a loading control to compare WT and PAD2 KO cell lysate and supernatant samples. Further, ATP stimulation also induced release of the PAD2 enzyme itself (Fig. 8). These data suggest that at least a subset of PAD2 substrates in BMMC are secreted proteins, and that PAD2 may have a yet uncharacterized extracellular function.

Discussion

Mast cells reside in the tissues, in close proximity to blood vessels and nerves, and secrete a number of effector molecules in the degranulation process (47). We find that PAD2 is expressed in mast cells, and that ATP induces P2X7-dependent protein citrullination in BMMC. To our knowledge, ATP is the first reported physiological stimulus of PAD2 activity.

P2X7 is expressed within the CNS and on cells of hematopoietic lineage (7). In this article, we describe the expression and functional activity of P2X7 in BMMC, which correlates with similar findings using primary human lung mast cells and the LAD2 human mast cell line (48, 49). We note that the presence and activity of P2X7 on BMMC has been previously described, although these reports have been subject to recent controversy (50).

Stimulation of the P2X7 is known to induce Ca²⁺ flux and downstream signaling events, such as protein phosphorylation and proteolytic cleavage (6). We found that ATP-induced PAD2 activation requires extracellular calcium, and that interference with p38 MAPK or PKC activation results in a significant reduction in BMMC citrullination, suggesting that P2X7-induced PAD2 activation lies downstream of p38 MAPK and PKC kinases. PKCγ can directly bind P2X7, and antisense oligo-mediated knockdown of PKCγ expression reduced ATP/P2X7-mediated Ca²⁺ influx (27). Because PAD2 requires high Ca²⁺ concentrations, the sensitivity of PAD2 activation to PKC inhibitors could be because of impaired Ca²⁺ flux (13, 27). However, PAD2 bears several putative PKCα, β1, and δ consensus sites; therefore, these PKC isoforms may be direct regulators of PAD2 activity (51). In contrast, PAD2 does not contain amino acid sequences that are ideal for p38 MAPK-mediated phosphorylation, likely signifying an indirect regulation of PAD2 by p38 MAPK (51). Because p38 MAPK phosphorylation was comparable in WT and PAD2−/− BMMC, we concluded that PAD2 activation follows, rather than precedes, p38 MAPK activation. Thus, in addition to Ca²⁺ mobilization, our studies suggest that P2X7-induced PKC and p38 MAPK signals converge to activate PAD2 in BMMC.

FIGURE 6. ATP-induced degranulation and cytokine secretion are normal in PAD2−/− BMMC. (A) WT (dark gray bars) or the PAD2−/− (light gray bars) BMMC were left unstimulated (control) or were stimulated with ATP (1 mM) or 50 ng/ml PMA and 1 μM ionomycin (PMA/I) for 1 h at 37°C. Percentage of β-hexosaminidase activity in the cell supernatants was measured in comparison with total β-hexosaminidase levels in the supernatants and lysates. WT, PAD2−/−, or P2X7−/− BMMC were stimulated with ATP (1 mM) or vehicle (control) for 24 h at 37°C. Cytokine levels of IL-6 (B), IL-13 (C), and TNF-α (D) were determined in the supernatants by ELISA. Representatives of three or more independent experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001.
Little is known about the physiological function of PAD2. Downstream events after ATP-induced P2X7 ligation are linked with the inflammatory response (23). Stimulation with ATP induces a P2X7-specific gene expression program (52). We found that Adamts-9, Rab6b, and TNFR2 transcript levels were diminished in ATP-stimulated mast cells devoid of PAD2. Proteolytic events are activated downstream of the P2X7, such as the maturation of IL-1β via caspase-1 activation or the release of the lysosomal protease cathepsin B (26, 53). Adamts-9 is also a protease thought to contribute to inflammatory arthritis by targeting the glycosaminoglycan region of aggrecan in the cartilage matrix (37). In mast cells, we find that ATP stimulation induces the expression of Adamts-9 in a PAD2-sensitive manner. Notably, P2X7−/− mice exhibit less severe cartilage damage in the collagen Ab-induced arthritis model (54). Rab6b, another PAD2-dependent ATP-induced target identified in our study, is a small GTPase that regulates retrograde vesicle transfer relevant to many cellular trafficking events induced by P2X7 activation (38, 40). Finally, ATP stimulation leads to upregulation of TNFR2 expression, which is diminished in PAD2−/− BMMC. The link between PAD2 and TNFR2 expression is particularly intriguing because polymorphisms in TNFR1 have been linked to disease severity in RA (36). Although the identity of the transcription factors downstream of PAD2 activation is currently unknown, these data point to a role for PAD2 in regulating mast cell gene expression.

The PAD2 enzyme and citrullinated proteins are found within the synovial fluid of RA patients (41). This is in opposition to mouse models of arthritis where the PAD2 message is abundant, but the PAD2 protein is absent from inflamed synovial tissue (55). In this study, we find that mast cell P2X7 activation leads to the release of citrullinated proteins and the PAD2 enzyme. We have not yet identified the PAD2 targets that are released by mast cells, but several known substrates of PAD enzymes reside in the extracellular space. Purified PAD2 and PAD4 can inactivate antithrombin through citrullination, and citrullinated antithrombin is present in the synovial fluid of RA patients (56). In addition, fibrinogen, a glycoprotein that is cleaved by thrombin, is also a known PAD target and a citrullinated autoantigen for RA (57, 58). Citrullination of several chemokines has been demonstrated in vitro and has been shown to negatively regulate chemokine activity in neutrophil and T cell recruitment (59–61). Because mast cells can produce chemokines and P2X7-dependent chemokine expression has been demonstrated in microglial cells, chemokines could be PAD2 targets after P2X7 stimulation (33, 62, 63).

The mechanism by which PAD2 and citrullinated proteins are released into the extracellular space by ATP is unknown. Notably, inhibition of apoptosis or necrosis did not interfere with ATP-induced citrullination. Stimulation with ATP leads to the release of mast cell secretory granules (29, 64). The PAD2 amino acid sequence does not contain a signal sequence, making it unlikely that PAD2 is targeted through the Golgi and the conventional secretory pathway into the secretory granules (65). However, the secretory granule network is connected with the endocytic network in mast cells, and secretory granules can receive cargo in post-Golgi events (64). Thus, PAD2 could enter into the granule

![FIGURE 7](image-url)  
**FIGURE 7.** ATP-induced upregulation in expression of Adamts-9, Rab6b, and TNFR2 requires PAD2 in BMMC. WT or PAD2−/− BMMC were stimulated with ATP (1 mM) or vehicle (control) for 3 h at 37°C. Total RNA was extracted and cDNA prepared. qPCR was performed as described in Materials and Methods to detect TNFR2 (A), Adamts-9 (B), or Rab6 (C) expression. Averages from three different cultures are shown.

![FIGURE 8](image-url)  
**FIGURE 8.** Citrullinated proteins and PAD2 are released from ATP-stimulated BMMC. WT or PAD2−/− BMMC were stimulated with 1 mM ATP (+) or vehicle (−) for 10 min. Cell supernatants were collected and cell extracts were prepared as described in Materials and Methods. Cell extracts (whole-cell lysates) and supernatants were subjected to SDS-PAGE and immunoblot analysis using the Abs specific for citrullinated proteins, PAD2, or tubulin. A representative of three or more independent experiments is shown.
compartment in this immunology. Although they are secreted proteins, members of the IL-1 cytokine family also lack a signal sequence (66, 67). Engagement of P2X7 on LPS-primed macrophages leads to inflammasome activation, processing of pro–IL-1ß, and release of mature IL-1ß (26). P2X7 stimulation was also reported to induce rapid plasma membrane shedding of IL-1ß–containing microvesicles (68) and to trigger a lysosomal secretion pathway presumably responsible for cathepsin secretion, which may also contribute to IL-1ß release (54, 69). Further, endosome-derived multivesicular bodies and released exosomes are also proposed as a mechanism for P2X7-induced IL-1ß release by murine macrophages (42). Finally, Takenouchi et al. (70) described a pathway leading to release of autophagolysosome content after P2X7 activation in microglial cells. Therefore, many potential pathways would provide extracellular access to PAD2 after ATP stimulation of mast cells.

Ablant PAD activity has been implicated in both the priming and effector phases of arthritis in mouse models of the disease. Using a genome-wide screen of mice, the region of murine chromosome 4 containing all PAD genes was recently shown to be linked to arthritis severity in a serum transfer model of arthritis, with the highest associated single nucleotide polymorphisms being within the PAD2 gene (71). Willis et al. (72) recently demonstrated that the general PAD inhibitor CI-amidine provides therapeutic benefit in the murine collagen-induced arthritis model. In human RA patients, ACPA are sensitive diagnostic markers that can appear before disease onset, correlate with the most erosive form of RA, and are predictive of disease progression (11, 73). Although variants of PAD4 are linked to RA in several Japanese and Korean cohorts, this association has not held up in most North American and European study groups, despite the prevalence of ACPA in all ethnic groups (74). Conversely, PAD2 expression is linked with higher systemic and local ACPA levels, and the PAD2 enzyme is expressed in the synovium of RA patients (12, 75). Despite the impressive body of literature implicating PAD2 and PAD4 in RA pathogenesis, the signals that induce Ca2+ flux, and thereby activate PAD activity in RA, are unknown.

Our studies reveal that PAD2 is also abundantly expressed in primary murine mast cell cultures, and the well-characterized inflammatory “danger” signal ATP induces robust PAD2- and P2X7-dependent protein citrullination in mast cells, a cell type linked to arthritis (18). Because P2X7-deficient mice exhibit reduced susceptibility and severity of disease in an Ab-induced arthritis model, it will be important to determine whether PAD2 contributes to P2X7-dependent disease pathogenesis by subjecting PAD2−/− mice to models of inflammatory arthritis (55, 76). We propose that ATP-induced activation of PAD2 during inflammation and the subsequent release of both citrullinated proteins and the PAD2 enzyme may promote the exposure of citrullinated self-Ags to the adaptive immune system, leading to the formation of ACPIA. Thus, targeting PAD2 may provide a novel therapeutic strategy to reduce the formation of citrullinated epitopes and to attenuate inflammatory arthritides, such as RA.

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