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The UDP-sugar-sensing $P2Y_{14}$ receptor promotes Rho-mediated signaling and chemotaxis in human neutrophils

Juliana I. Sesma,[1] Silvia M. Kreda,[1] Natacha Steinckwich-Besancon,[2] Hong Dang,[1] Rafael García-Mata,[3] T. Kendall Harden,[4] and Eduardo R. Lazarowski[1]

[1]*Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina;* [3]*Department of Cell and Developmental Biology, University of North Carolina School of Medicine, Chapel Hill, North Carolina;* [4]*Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina; and* [2]*Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina*

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Sesma JI, Kreda SM, Steinckwich-Besancon N, Dang H, García-Mata R, Harden TK, Lazarowski ER. The UDP-sugar-sensing $P2Y_{14}$ receptor promotes Rho-mediated signaling and chemotaxis in human neutrophils. *Am J Physiol Cell Physiol* 303: C490–C498, 2012. First published June 6, 2012; doi:10.1152/ajpcell.00138.2012.—The G_i-coupled $P2Y_{14}$ receptor ($P2Y_{14}$-R) is potently activated by UDP-sugars and UDP. Although $P2Y_{14}$-R mRNA is prominently expressed in circulating neutrophils, the signaling pathways and functional responses associated with this receptor are undefined. In this study, we illustrate that incubation of isolated human neutrophils with UDP-glucose resulted in cytoskeleton rearrangement, change of cell shape, and enhanced cell migration. We also demonstrate that UDP-glucose promotes rapid, robust, and concentration-dependent activation of RhoA in these cells. Ecto-nucleotidases expressed on neutrophils rapidly hydrolyzed extracellular ATP, but incubation with UDP-glucose for up to 1 h resulted in negligible metabolism of the nucleotide-sugar. HL60 human promyelocytic leukemia cells do not express the $P2Y_{14}$-R, but neutrophil differentiation of HL60 cells with DMSO resulted in markedly enhanced $P2Y_{14}$-R expression. Accordingly, UDP-glucose, UDP-galactose, and UDP-*N*-acetylglucosamine promoted Rho activation in differentiated but not in undifferentiated HL60 cells. Stable expression of recombinant human $P2Y_{14}$-R conferred UDP-sugar-promoted responses to undifferentiated HL60 cells. UDP-glucose-promoted RhoA activation also was accompanied by enhanced cell migration in differentiated HL60 cells, and these responses were blocked by Rho kinase inhibitors. These results support the notion that UDP-glucose is a stable and potent proinflammatory mediator that promotes $P2Y_{14}$-R-mediated neutrophil motility via Rho/Rho kinase activation.

purinergic receptors

THE $P2Y_{14}$ RECEPTOR ($P2Y_{14}$-R) was identified as a G_i-coupled receptor that is potently and selectively activated by UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine (6). UDP also potently activates the $P2Y_{14}$-R (5), but UTP, ATP, ADP, and other naturally occurring di- or triphosphate nucleotides are not agonists at this receptor (5, 6).

$P2Y_{14}$-R mRNA is expressed throughout the brain (6) and was markedly upregulated after immunological challenge with lipopolysaccharide (37). $P2Y_{14}$-R also is expressed at the mRNA level in several peripheral tissues, including immune-competent cells (21). Relatively high $P2Y_{14}$-R levels were observed in immature human dendritic cells that mature and migrate to sites of inflammation (47), in bone marrow hematopoietic cells, where it was proposed to mediate chemotaxis (33), and in RBL-2H3 mast cells, where UDP-glucose promoted Ca^{2+} mobilization, phosphorylation of ERK1/2, and β-hexosaminidase release (18).

The high level of expression of $P2Y_{14}$-R in leucocytes and other immune cells supports a potential role for this receptor in immune system homeostasis, but association of UDP-glucose effects with $P2Y_{14}$-R activity has been difficult to establish in these tissues. Of particular relevance to inflammation, circulating polymorphonuclear neutrophils (PMNs) are the peripheral human cell type that most abundantly expresses $P2Y_{14}$-R mRNA (37), but the functional consequences of $P2Y_{14}$-R activation in PMNs remain undefined. Scrivens and Dickenson (43) reported that UDP-glucose promoted a modest inhibition of forskolin-stimulated cyclic AMP formation in neutrophils, but other UDP-sugars known to promote $P2Y_{14}$-R activation had no effect (43). UDP-glucose also evoked a modest increase in ERK phosphorylation in these cells, but this response was observed only at high micromolar/submillimolar concentrations and was not mimicked by other UDP-sugars (43). UDP-glucose failed to induce elastase secretion from human PMNs (43). Thus, unambiguous association of the expression of $P2Y_{14}$-R transcripts with $P2Y_{14}$-R-mediated responses in PMNs remains to be established.

In the present study, we used isolated human neutrophils and neutrophil-like HL60 human promyelocytic leukemia cells to illustrate that UDP-glucose evokes activation of Rho GTPases in a $P2Y_{14}$-R expression-dependent manner. Rho activation was accompanied by cytoskeleton remodeling and enhanced cell motility.[1]

MATERIALS AND METHODS

Human neutrophils and cell cultures. Peripheral neutrophils were isolated from fresh venous blood samples from healthy volunteers with written informed consent and the approval of the University of North Carolina Institutional Review Board (IRB). Neutrophils were isolated (>98% purity) using Ficoll-paque Plus (GE Healthcare) and 3% dextran as described previously (29, 52). HL60 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum at a cell density of 3×10^5 cells/ml, as previously described (16). Neutrophil differentiation of HL60 (dHL60) cells was achieved by

Address for reprint requests and other correspondence: E. R. Lazarowski, Cystic Fibrosis/Pulmonary Research and Treatment Center, 7017 Thurston-Bowles Bldg., CB 7248, Univ. of North Carolina. Chapel Hill, NC 27599-7248 (e-mail: eduardo_lazarowski@med.unc.edu).

[1] This article is the topic of an Editorial Focus by Scott M. O'Grady (40a).

inclusion of 1.3% DMSO in the culture medium for 5 days (16). Neutrophils and HL60 cells were rinsed and, except when indicated otherwise, cells were incubated (1–10 $\times$ 10^6 cells/ml) in HEPES-buffered (pH 7.4) Hanks' balanced salt solution supplemented with 1.6 mM $CaCl_2$ and 0.8 mM $MgCl_2$ (HBSS). Apyrase (1 U/ml) was added to PMN suspensions during the isolation steps. Inclusion of apyrase ensures the removal of ATP and UTP potentially released from damaged or mechanically activated neutrophils, e.g., during cell washes and centrifugations (28), and thus, avoids unwanted activation of $P2Y_2$ or P2X1 receptors expressed in these cells (32, 51, 52). HPLC analysis verified that 100 μM ATP and 100 μM UTP were completely hydrolyzed by 1 U/ml apyrase in <5 min, while 100 μM UDP-glucose remained essentially unchanged after 90 min (not shown).

Extracellular metabolism of UDP-glucose and ATP. Neutrophils (2×10^6/ml) were incubated in HBSS (supplemented as above) in the presence of 100 μM UDP-glucose or 100 μM ATP, or in the presence of 0.1 μCi UDP-[^{3}H]glucose (26 Ci/mmol) or 0.1 μCi [^{3}H]ATP (37 Ci/mmol). At the times indicated, cells were centrifuged and nucleotides present in the supernatant were separated by HPLC (Waters) via a Nova-Pack C18 column. Absorbance at $\lambda = 260$ nm and radioactivity were quantified on-line as previously described (27, 31).

Stable expression of $P2Y_{14}$-R in HL60 cells. Retroviruses encoding the human $P2Y_{14}$-R were produced using the pLXSN expression vector, as previously described (16). HL60 cells were infected with $P2Y_{14}$-R-bearing retroviruses and selected in medium containing 0.5 mg/ml G418.

RhoA pull-down assays. GTP-bound RhoA was measured using the Rho Activation Assay Biochem Kit, following the manufacturer's instructions. Briefly, neutrophils or 12-h serum-starved HL60 cells were rinsed and suspended in HBSS (10^7 cells/ml), preincubated for 45 min at 37°C, and incubated for 1 min (or the indicated times) with vehicle or agonists. Incubations were terminated by addition of lysis buffer (supplied in the kit) followed by rapid centrifugation, and aliquots of supernatants were incubated with Rhotekin-RBD beads for 1 h at 4°C. RhoGTP from pull-down assays and total RhoA were resolved by SDS-PAGE and analyzed by Western blotting, as previously described (45). Apyrase (1 U/ml) was added to the neutrophil suspension during these incubations.

Chemotaxis. Cells were loaded with 2.5 μM carbocyanide dye DID (Vybrant Cell-Labeling Solutions, Molecular Probes) for 15 min (10^6 cells/ml) in RPMI medium (PMN) or HBSS (HL60 cells) supplemented with 10% heat-inactivated fetal bovine serum, rinsed twice, and suspended in RPMI or HBSS, as above. Chemotaxis was quantified in 96-multiwell FluoroBlock insert (BD Falcon), following the manufacturer's instructions. Briefly, vehicle or agonist was added to the lower compartment (225 μl), and 75-μl of a cell suspension (2×10^5 cells) was loaded onto the upper compartment, separated from the bottom by a 3-μm pore diameter polyethylene terephthalate (PEI) filter membrane (specially designed to block light transmission, simplifying detection of migrating fluorescently labeled cells). Agonists were added to both the lower and the upper compartments in experiments designed to assess random migration. The fluorescent signal (F, 612 nm excitation/670 nm emission) was read at the indicated times, using a Tecan Infinite M1000 plate reader. The chemotaxis index (Ctx Index) was defined as:

$$\text{Ctx Index} = (F_1 - F_B)/(F_0 - F_B)$$

where F_1 and F_0 represent the fluorescence signal from stimulated and nonstimulated cells, respectively, and F_B is background fluorescence measured in the absence of cells.

To measure the rate of diffusion of UDP-glucose through the filter membrane, 100 μM UDP-[^{3}H]glucose (200,000 counts per minute) was added to the lower compartment, and the radioactivity present in both the lower and the upper compartments was quantified at various times by liquid scintillation counting. UDP-glucose diffusion was calculated as the percentage of counts present at a given time (T) in the upper compartment, relative to $T = \infty$ (i.e., at equilibrium).

Staining of F-actin and confocal microscopy. Neutrophils (2×10^6) were incubated with agonist or vehicle for 15 min, fixed with 4% paraformaldehyde (5 min), rapidly centrifuged, and resuspended in 0.1% Triton X-100 for 20 min. Cells were stained with AlexaFluor488-phalloidin to label F-actin and propidium iodide to label the nuclei. Cells were cytospun and mounted on coverslips and viewed in a Leica SP5 confocal microscope, as previously described (24).

Reagents. UDP-glucose was purchased from Fluka (Sigma-Aldrich, St. Louis, MO). HPLC analysis of stock solutions of UDP-glucose indicated no detectable contamination with ATP or any other nucleotide signaling molecule (not shown). Apyrase and nucleotide pyrophosphatase from *Crotalus adamanteus* were also from Sigma. The RhoA activation Assay Biochem kits (including anti-RhoA antibody) were purchased from Cytoskeleton (Denver, CO). H1152, Y27632, and wortmannin were obtained from Calbiochem (La Jolla, CA). UDP-[^{3}H]glucose and [^{3}H]ATP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were of the highest purity available.

Data analysis. Statistical analysis was performed using ANOVA with post hoc Tukey honestly significant difference (HSD); $P \leq 0.05$ (JMP Genomics, version 4.1, SAS, Cary, NC).

RESULTS

Stability of UDP-glucose on human neutrophils. The capacity of human neutrophils to rapidly hydrolyze extracellular ATP and other nucleotides is well established (11, 25, 42), but the extent to which these cells metabolize UDP-glucose is not known. Thus, we quantified the stability of this nucleotide-sugar in neutrophil suspensions using HPLC analysis. Incubation of neutrophils with 100 μM UDP-glucose for 60 min resulted in negligible metabolism of the nucleotide-sugar (Fig. 1*A*), i.e., <1% of the initial UDP-glucose was recovered as UMP. In contrast, neutrophils rapidly hydrolyzed 100 μM ATP, displaying an apparent hydrolysis rate of $V_{hyd} = 7.3$ nmol/min $\times$ 10^6 cells (Fig. 1*A*). ADP and AMP were the major products of ATP metabolism (not shown). Stability of nucleotides in the presence of PMNs also was monitored using UDP-[^{3}H]glucose or [^{3}H]ATP as high specific activity radiotracers. As shown in Fig. 1*B*, [^{3}H]ATP decayed with an apparent half-life of 2.5 min, while UDP-[^{3}H]glucose remained essentially unchanged after 60 min. These results are in agreement with previous reports indicating that NTPDase1, which hydrolyzes ATP and other NTPs and NDPs, but not UDP-sugars, is expressed on the surface of human neutrophils (11, 25, 42). The data also indicate that little or no ecto-nucleotide pyrophosphatase activity (which hydrolyzes UDP-sugars) is present on these cells.

UDP-glucose promotes actin cytoskeleton reorganization, cell migration, and Rho activation in human neutrophils. We tested the hypothesis that UDP-glucose acts as an extracellular signaling molecule for neutrophils by first assessing its effect on cell shape and actin cytoskeleton reorganization, visualized via differential interference contrast (DIC) and fluorescence microscopy. In the absence of external stimulus, most neutrophils displayed round shape and phalloidin uniformly stained the cortical actin fibers (Fig. 2, *A* and *B*, *left*). Incubation of neutrophils for 15 min with 100 μM UDP-glucose resulted in changes in cell shape (i.e., from round to elongated shape) that were accompanied by a sharp increase in the intensity of the cortical actin staining and the formation of a broad ruffle-like structure in one cellular pole (Fig. 2, *A* and *B*, *right*). These morphological changes were observed in 56.0 ± 4% of the cells incubated with UDP-glucose, as opposed to 15.2 ± 3% in

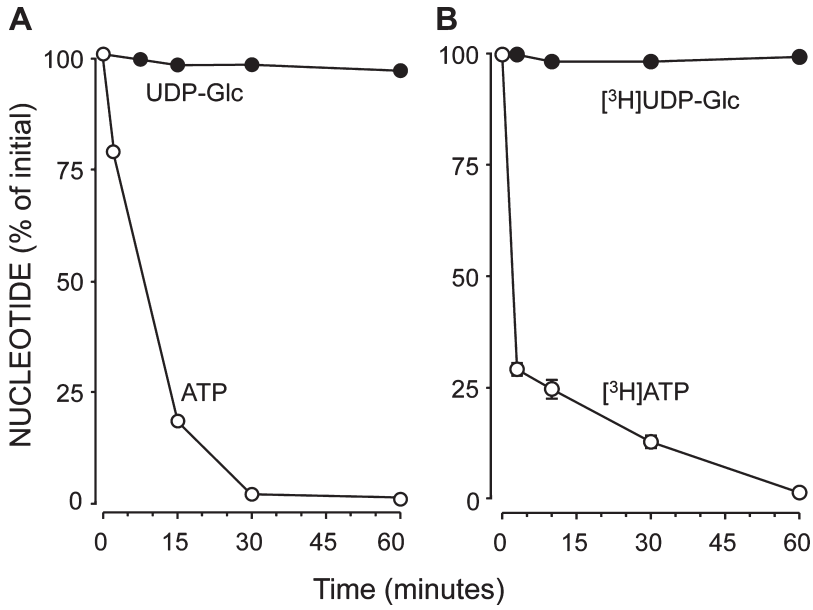

Fig. 1. Stability of UDP-glucose (UDP-Glc) in human neutrophils. Neutrophils were incubated for the indicated times with 100 μM UDP-glucose or ATP (*A*) or trace amounts (0.1 μCi) of UDP-[^{3}H]glucose or [^{3}H]ATP (*B*). Nucleotides were separated and quantified by HPLC. The data are representative of two independent experiments performed in duplicate.

untreated cells (means ± SE). The data suggest that UDP-glucose promotes changes in the organization of actin fibers in neutrophils.

Observation of UDP-glucose-dependent cell shape change and cytoskeleton rearrangement suggests that this nucleotide-sugar acts as an extracellular signaling molecule to regulate cellular functions associated with neutrophil motility. Studies using a modified Boyden chamber illustrated that UDP-glucose promotes PMN migration (Fig. 3). That is, enhanced cell migration was observed when UDP-glucose was added to the lower compartment of the chemotaxis chamber, but not when the concentration gradient of agonist was abolished by addition of UDP-glucose to both compartments (Fig. 3*A*). Thus, UDP-glucose enhances neutrophil chemotaxis but has no effect on random migration as illustrated by its lack of effect in the absence of a concentration gradient. In agreement with our HPLC analysis indicating that UDP-glucose stock solutions contained no detectable ATP or UTP (not shown), chemotaxis was not affected by inclusion of apyrase in the lower compartment of the chamber (Fig. 3*B*). However, UDP-glucose-evoked

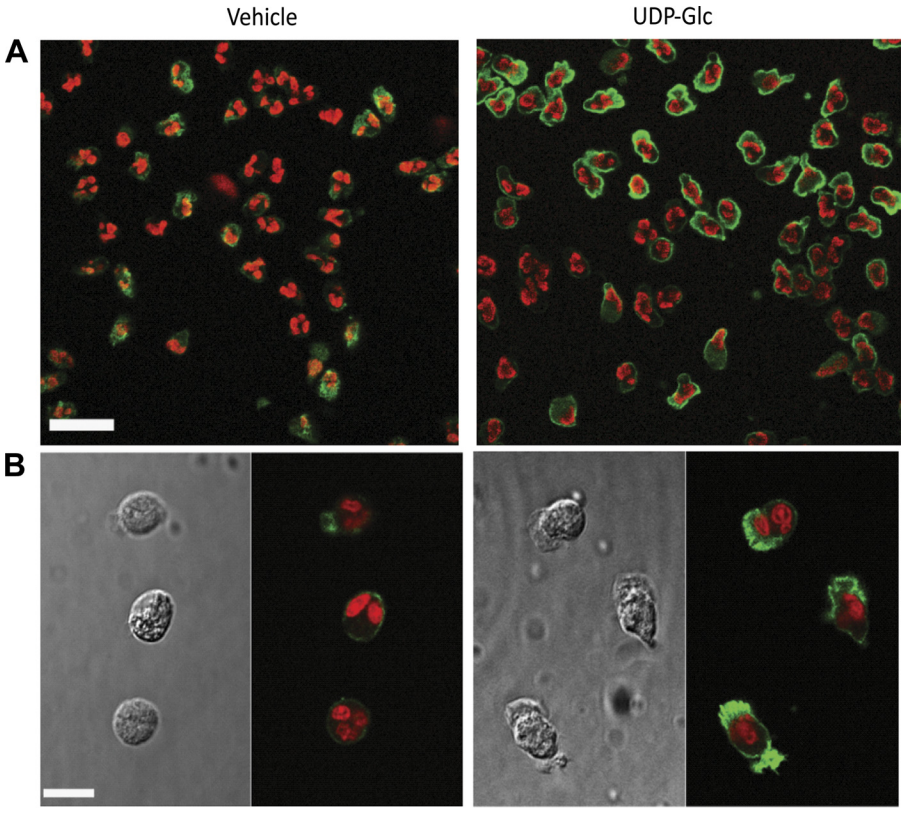

Fig. 2. UDP-glucose induces cell shape changes and cytoskeleton remodeling in human neutrophils. Neutrophils were incubated for 15 min with vehicle or 100 μM UDP-glucose. Cells were fixed and stained with fluorescent phalloidin (actin cytoskeleton) and propidium iodide (nucleus) as described in MATERIALS AND METHODS. *A*: overlay of confocal planes representing actin and nuclear staining of neutrophils. Bar, 25 μm. *B*: overlay of confocal planes representing actin and nuclear staining and the corresponding differential interference contrast confocal plane (gray images) of neutrophils at high-power magnification. Bar, 10 μm. The images are representative of two separate experiments in which 15–20 fields were randomly analyzed for each condition.

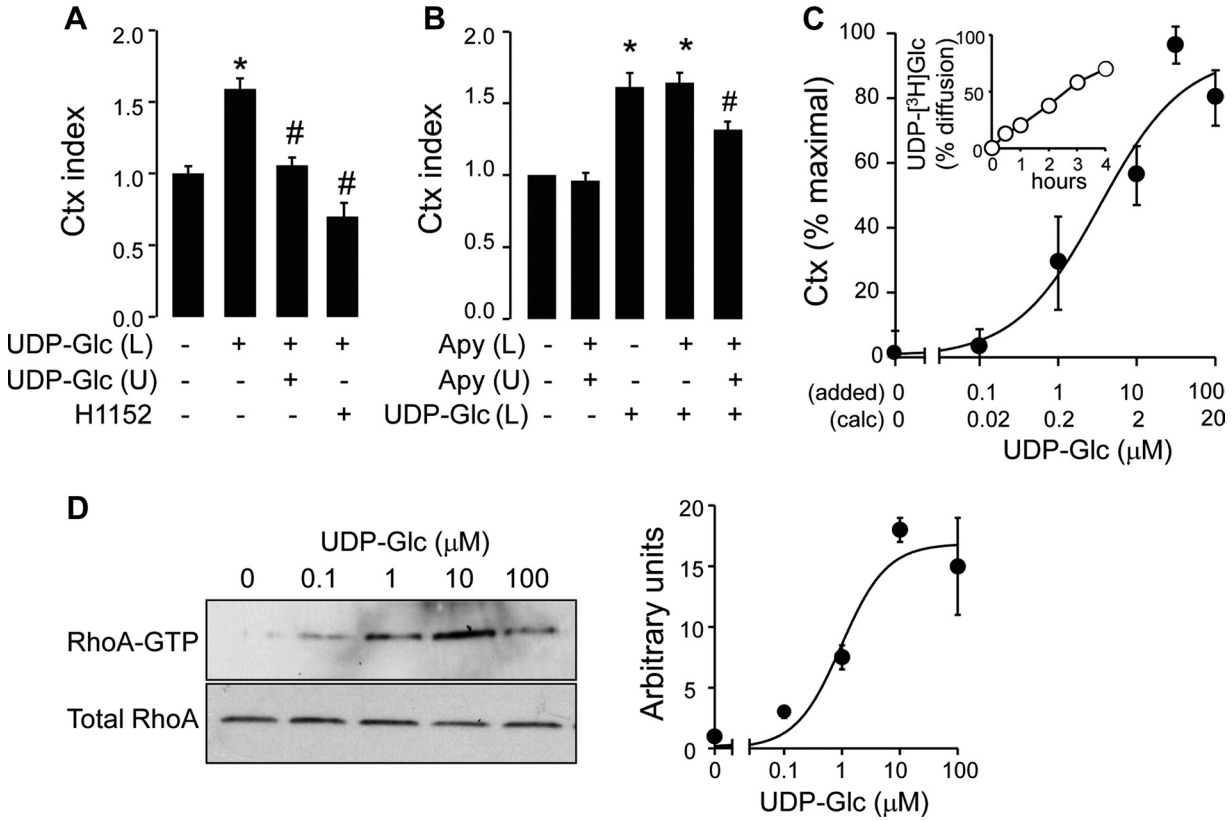

Fig. 3. UDP-glucose promotes chemotaxis (Ctx) and RhoA activation in human neutrophils. *A*: neutrophil migration in response to vehicle or 100 μM UDP-glucose added to either the lower (L) or the upper (U) compartment of a Boyden chamber. As indicated, neutrophils were preincubated for 15 min with 1 μM H1152. Results are expressed as chemotaxis index (see MATERIALS AND METHODS) and are means ± SE from three separate experiments, each one performed in quadruplicate. *,#Significantly different from vehicle and UDP-glucose (lower), respectively, $P < 0.05$ by 2-way ANOVA. *B*: neutrophil migration in response to vehicle or 100 μM UDP-glucose added to the lower compartment. Apyrase (Apy; 5 U/ml) was included in the lower and/or upper compartment, as indicated. Results are means ± SE from two separate experiments, each one performed in quadruplicate. *,#Significantly different from vehicle and UDP-glucose without apyrase, respectively, $P < 0.05$ by 2-way ANOVA. *C*: concentration-effect relationship for UDP-glucose-promoted neutrophil chemotaxis. The data (means ± SE, $n = 4$) are representative of two independent experiments. The values on the *x*-axis indicate the concentration of agonist added to the lower compartment (added), and the concentration of agonist calculated (calc) to reach the upper compartment at the time of measurement (see *inset*). *Inset*: time course for the diffusion of 100 μM UDP-[^{3}H]glucose through the filter membrane (means ± SD, $n = 3$; representative of two experiments). *D*: RhoA activation was measured in neutrophils that were incubated for 1 min in the presence of the indicated concentrations of UDP-glucose. The data are presented as means ± SE of results from three separate experiments.

chemotaxis was partially reduced when apyrase was added to both lower and upper compartments, suggesting that ATP/UTP released from neutrophils contributed, at least in part, to this response.

Chemotaxis was observed in response to the addition of 1 μM UDP-glucose and was maximal with 30 μM UDP-glucose (Fig. 3*C*). These nominal concentration values overestimate the actual concentration of agonist reaching the cells, since only ~20% of the UDP-glucose added to the lower compartment was recovered in the upper compartment after 60 min (Fig. 3*C*, *inset*). Based on the concentration of agonist estimated to reach the upper chamber at the time of the chemotaxis measurement, UDP-glucose promoted neutrophil chemotaxis with potency ($EC_{50} = 0.7$ μM; Fig. 3*C*) similar to that observed for activation of the $P2Y_{14}$-R in a variety of test systems (21).

Members of the Rho family of GTPases play critical roles in the cytoskeletal remodeling response associated with neutrophil motility (39, 40). Rho-associated kinase also is important for motile function of neutrophils (39), and UDP-glucose-promoted PMN migration was completely inhibited by the Rho kinase inhibitor H1152 (1 μM) (Fig. 3*A*).

We used the Rhotekin-RBD pull-down assay to examine the extent to which UDP-glucose promotes activation of RhoA in PMNs. Addition of UDP-glucose to isolated neutrophils resulted in rapid (1 min) formation of RhoA-GTP, which was evident with 100 nM UDP-glucose and was maximal with 10 μM UDP-glucose ($EC_{50} = 0.9$ μM; Fig. 3*D*), consistent with UDP-glucose acting through the $P2Y_{14}$-R (21).

Collectively, the data in Figs. 1–3 indicate that UDP-glucose is a potent and highly stable agonist of the $P2Y_{14}$-R endogenously expressed by human neutrophils. Activation of this G protein-coupled receptor promotes RhoA activation, cell shape changes, and chemotaxis in these cells.

UDP-glucose promotes $P2Y_{14}$-R-mediated activation of RhoA in HL60 cells. To assess more extensively the involvement of $P2Y_{14}$-R in UDP-glucose-evoked RhoA activation in neutrophils, studies were conducted using neutrophil-like HL60 human promyelocytic leukemia cells. We previously reported that undifferentiated HL60 cells do not express $P2Y_{14}$-R transcripts (5, 16), and as is illustrated in Fig. 4*A*, these cells also did not respond to UDP-glucose with an increase in RhoA-GTP formation. UDP-galactose and UDP-*N*-acetylglucosamine also had no effect on RhoA activation (not

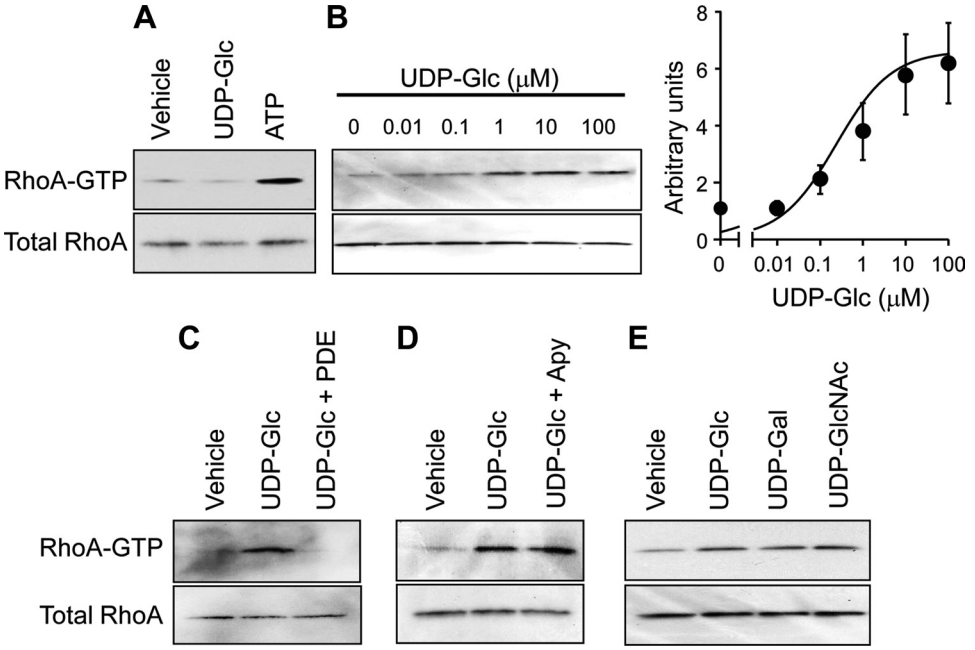

Fig. 4. UDP-sugars promote RhoA activation in differentiated HL60 (dHL60) cells. *A*: RhoA activation was measured in undifferentiated HL60 cells incubated for 1 min with vehicle, 100 μM UDP-glucose, or 100 μM ATP. *B*: concentration-effect relationship for UDP-glucose-evoked RhoA activation in dHL60 cells. Error bars indicate differences to the mean from two independent experiments. *C*: the effect of 100 μM UDP-glucose on dHL60 cells was abolished by pretreatment (15 min) of the nucleotide-sugar with 1 U/ml nucleotide-pyrophosphatase [a phosphodiesterase (PDE)]. *D*: apyrase (1 U/ml, 15 min) had no effect on UDP-glucose-evoked RhoA activation in dHL60 cells. *E*: RhoA activation was measured in dHL60 cells incubated with vehicle or 100 μM UDP-glucose, UDP-galactose (UDP-Gal), or UDP-*N*-acetyl-glucosamine (UDP-GlcNAc). All incubations (*A–E*) were for 1 min.

shown), but robust formation of RhoA-GTP occurred in the presence of ATP (Fig. 4*A*), likely reflecting activation of endogenous P2X1 and/or $P2Y_2$ receptors (4, 5, 10, 52).

Differentiation of HL60 cells with DMSO (dHL60 cells) results in $P2Y_{14}$-R mRNA expression (5, 16), and as is illustrated in Fig. 4*B*, RhoA activation in dHL60 cells was observed with low micromolar concentrations of UDP-glucose (EC_{50} = 0.7 μM), consistent with the concentration dependence of a $P2Y_{14}$-R. To support the conclusion that activation of RhoA occurs through an extracellular signaling action of UDP-glucose, the effects of *Crotalus adamanteus* nucleotide pyrophosphatase, a phosphodiesterase (PDE) that hydrolyzes nucleotide-sugars and nucleotides (31), and apyrase, which hydrolyzes nucleotides but not nucleotide-sugars (31), were examined. Activation of RhoA in the presence of UDP-glucose was virtually eliminated by preincubation of UDP-glucose with 1 U/ml PDE (Fig. 4*C*), but not by 1 U/ml apyrase (Fig. 4*D*). UDP-galactose and UDP-*N*-acetylglucosamine also promoted RhoA activation in dHL60 cells (Fig. 4*E*).

To further assess the involvement of the $P2Y_{14}$-R in UDP-glucose-evoked RhoA activation in HL60 cells, the human $P2Y_{14}$-R was stably expressed in these cells ($P2Y_{14}$-HL60 cells) via retroviral infection (16). In contrast to the lack of effect observed in undifferentiated HL60 cells (see Fig. 4*A*), UDP-glucose promoted RhoA activation in undifferentiated $P2Y_{14}$-HL60 cells (Fig. 5). UDP-glucose-evoked RhoA activation was robust between 1 and 15 min following agonist addition, and it faded after 30 min (Fig. 5*A*). UDP-glucose-promoted RhoA activation in $P2Y_{14}$-HL60 cells exhibited an EC_{50} value of 0.4 μM (Fig. 5*B*). UDP-galactose and UDP-*N*-acetylglucosamine also promoted RhoA-GTP formation in undifferentiated $P2Y_{14}$-HL60 cells (Fig. 5*C*).

Pertussis toxin and phosphoinositide 3-kinase inhibition abolish P2Y14-R-promoted Rho activation in HL60 cells. Several studies from our and other groups support the notion that the human $P2Y_{14}$-R signals through G_i (6, 15–17, 31). In agreement with this concept, preincubation of dHL60 (Fig. 6*A*) with pertussis toxin abolished UDP-glucose-promoted RhoA

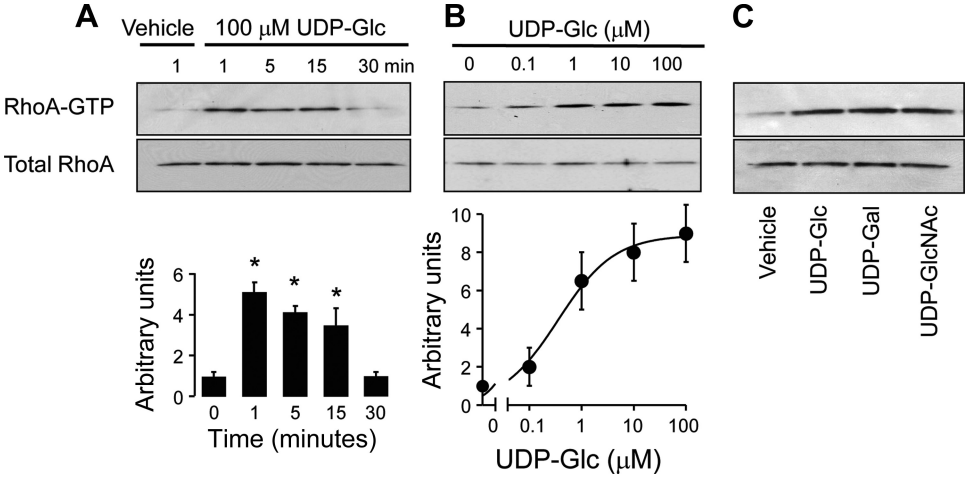

Fig. 5. $P2Y_{14}$ receptor ($P2Y_{14}$-R) confers UDP-sugar-promoted RhoA activation to undifferentiated HL60 cells. *A*: undifferentiated $P2Y_{14}$-HL60 cells were incubated for the time indicated with 100 μM UDP-glucose. The data are presented as means ± SE of results from three separate experiments. *Significantly different from vehicle, $P < 0.05$ by 2-way ANOVA. *B*: cells were incubated for 1 min with the indicated concentrations of UDP-glucose. The data are presented as means ± SE of results from three separate experiments. *C*: cells were incubated for 1 min with vehicle or 100 μM of UDP-glucose, UDP-galactose, or UDP-*N*-acetyl-glucosamine; the results are representative of three experiments.

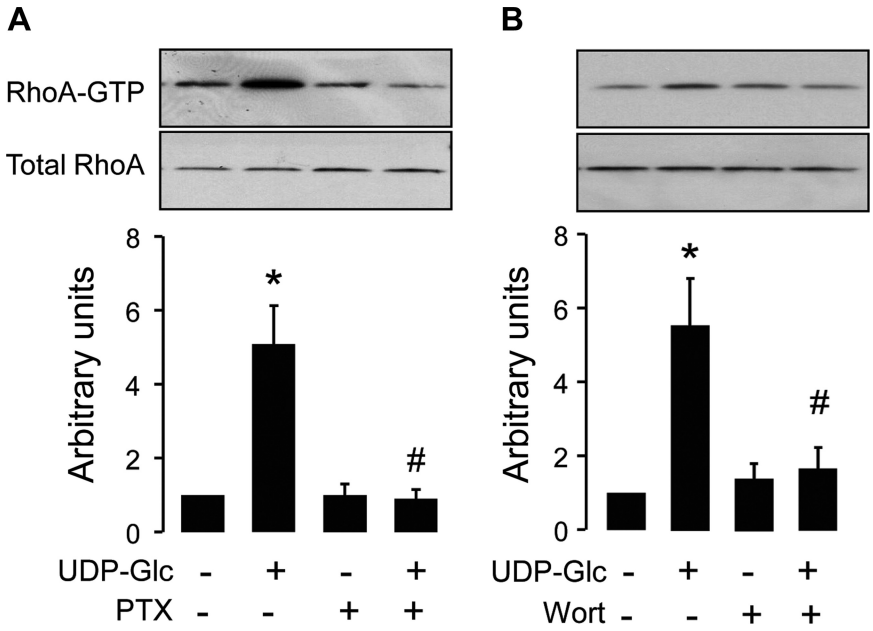

Fig. 6. Pertussis toxin (PTX) and a phosphoinositide 3-kinase inhibitor abolish $P2Y_{14}$-R-promoted Rho activation in HL60 cells. dHL60 cells were preincubated overnight with vehicle or 100 ng/ml of pertussis toxin (*A*) or 15 min with vehicle or 100 nM wortmannin (Wort) (*B*) and stimulated for 1 min with 100 μM UDP-glucose. The data are presented as means ± SE of results from four separate experiments. *,#Significantly different from vehicle and UDP-glucose, respectively, $P < 0.05$ by 2-way ANOVA.

activation. A possible mechanism whereby G_i-coupled receptors promote RhoA activation in HL60 cells involves activation of phosphoinositide 3-kinase (PI-3-kinase) (44). Consistent with this hypothesis, incubation of dHL60 cells with wortmannin, a selective inhibitor of PI-3-kinase, abolished UDP-glucose-promoted RhoA activation (Fig. 6*B*).

UDP-glucose promotes dHL60 cell migration. Incubation of dHL60 cells with 100 μM UDP-glucose (added to the lower compartment of the Boyden chamber) resulted in enhanced cell migration. As shown above with neutrophils, cell migration was not observed when the concentration gradient of agonist was abolished by addition of UDP-glucose to both compartments of the chamber, and it was inhibited by the Rho kinase inhibitors H1152 and Y27632 (Fig. 7*A*). As predicted, UDP-glucose-evoked dHL60 cell migration was abolished by preincubation of UDP-glucose with nucleotide pyrophosphatase, but not by apyrase (Fig. 7*A*). Addition of apyrase to the lower compartment of the chamber had no effect on dHL60 cell chemotaxis, but UDP-glucose-evoked chemotaxis was partially reduced when apyrase was also present in the upper chamber (Fig. 7*B*). The concentration-effect relationship for UDP-glucose-promoted chemotaxis in dHL60 is illustrated in Fig. 7*C*. Based on the diffusion rate of agonist through the chemotaxis membrane (see Fig. 3*C*), the estimated EC_{50} value for UDP-glucose-promoted dHL60 cell chemotaxis was 0.5 μM (Fig. 7*C*).

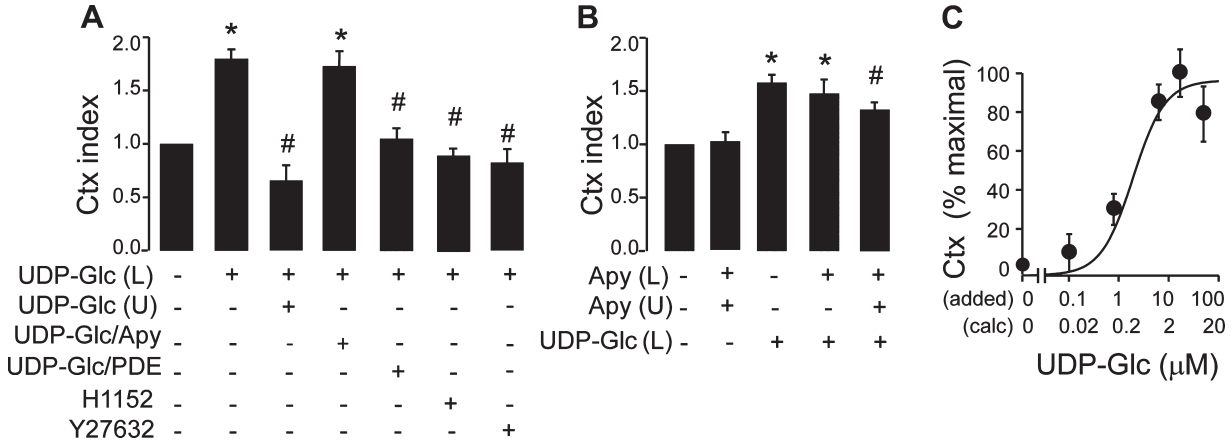

Fig. 7. UDP-glucose promotes chemotaxis in dHL60 cells. *A*: chemotaxis of dHL60 cells was measured in response to vehicle or 100 μM UDP-glucose added either to the lower or to the upper compartment of the Boyden chamber, as indicated. UDP-glucose was preincubated with 1 U/ml of apyrase or nucleotide pyrophosphatase (PDE) for 15 min before addition of the agonist to the lower compartment, and HL60 cells were preincubated with vehicle, 1 μM H1152, or 5 μM Y27632 for 15 min. Results are means ± SE from three separate experiments, each one performed in quadruplicate. *,#Significantly different from vehicle and UDP-glucose (lower), respectively, $P < 0.05$ by 2-way ANOVA. *B*: dHL60 cells migration in response to vehicle or 100 μM UDP-glucose added to the lower compartment. Apyrase (5 U/ml) was included in the lower and/or upper compartment, as indicated. Results are means ± SE from three separate experiments, each one performed in sextuplicate. *,#Significantly different from vehicle and UDP-glucose without apyrase, respectively, $P < 0.05$ by 2-way ANOVA. *C*: concentration-effect relationship for UDP-glucose-promoted chemotaxis in dHL60 cells; the values on the *x*-axis indicate the concentration of agonist added to the lower compartment (added), and the concentration of agonist calculated to reach the upper compartment (calc), as in Fig. 3*C*. The results represent means ± SE from two separate experiments, each performed in quadruplicate.

DISCUSSION

$P2Y_{14}$-R mRNA is highly abundant in circulating neutrophils (37), and UDP-glucose promotes ERK1/2 phosphorylation in these cells (43). Involvement of $P2Y_{14}$-R activation in neutrophil function has, nonetheless, remained poorly defined. In the present study, we demonstrate that addition of UDP-glucose to human neutrophils results in rapid and robust activation of RhoA, and this response occurs with concentration dependence consistent with a $P2Y_{14}$-R-mediated response. UDP-glucose-evoked Rho activation was accompanied by changes in cell shape (with the formation of ruffle-like structures in one cellular pole), cytoskeleton rearrangements, and enhanced neutrophil chemotaxis. Furthermore, using the HL60 human promyelocytic leukemia cells, we demonstrate that UDP-glucose-evoked responses occurred in a $P2Y_{14}$-R-dependent manner. That is, UDP-glucose had no effect on HL60 cells lacking $P2Y_{14}$-R mRNA expression, but strong UDP-glucose-evoked RhoA activity was observed after either inducing endogenous expression of the receptor via DMSO differentiation (dHL60 cells) or stably expressing the $P2Y_{14}$-R via retroviral infection ($P2Y_{14}$-HL60 cells).

A caution was introduced by Brautigam et al. (3) for studies of inflammatory and other effects potentially regulated by the $P2Y_{14}$-R. That is, although commercial sources of UDP-glucose induced inflammatory effects in N9 microglia, this action was only observed with high micromolar (>75 μM) concentrations of bacterially derived but not synthetic UDP-glucose; other nucleotide sugars such as UDP-galactose also had no effect (3). The conclusion that actions of UDP-sugars on neutrophils reflected activation of $P2Y_{14}$-R is firmly supported by the following observations: *1*) HPLC analysis of UDP-glucose indicated no detectable contamination with ATP or any other nucleotide signaling molecule; moreover, the nucleotide di- and tri-phosphohydrolase apyrase (1 U/ml) was included in the PMN suspension medium during the Rho assay to minimize neutrophil activation by ATP/UTP potentially released during PMN isolation (28); *2*) both RhoA activation and cell migration in the presence of UDP-glucose were virtually eliminated by preincubation of UDP-glucose with nucleotide pyrophosphatase, which hydrolyzes UDP-sugars (31), but not by apyrase; *3*) neutrophil activation occurred with concentrations of UDP-glucose consistent with $P2Y_{14}$-R activation; and *4*) UDP-glucose, UDP-galactose, and UDP-*N*-acetylglucosamine promoted RhoA activation in dHL60 cells, which natively express $P2Y_{14}$-R (16), and in undifferentiated HL60 cells expressing recombinant $P2Y_{14}$-R, but not in wild-type undifferentiated HL60 cells, which do not express $P2Y_{14}$-R (16).

Extensive literature associates $P2Y_{14}$-R-promoted responses with activation of heterotrimeric G proteins of the G_i subfamily (reviewed in Ref. 21). Our results illustrating that pertussis toxin abolishes UDP-glucose-promoted RhoA activation in dHL60 and $P2Y_{14}$-HL60 cells are consistent with this notion. We also showed that RhoA activation was inhibited by a selective inhibitor of PI-3-kinase, a downstream effector of G_i (20, 49). Based on these results, we propose that the $P2Y_{14}$-R promotes activation of G_i, resulting in $G\beta\gamma$-evoked PI-3-kinase-mediated formation of phosphatidylinositol(3,4,5)trisphosphate, recruitment of a Rho G protein exchange factor to the plasma membrane, and facilitation of RhoGTP formation. Such a signaling pathway was previously illustrated with formyl peptide-stimulated monocytes (7).

The present study provides evidence for a functional role of the neutrophil $P2Y_{14}$-R, i.e., modulation of neutrophil shape change and motility. Furthermore, our observation that Rho kinase inhibitors markedly reduced neutrophil motility are in line with studies indicating that activation of RhoA/Rho kinase results in myosin activation, rear contraction, and development of cell polarity in migrating neutrophils (32, 40, 54, 55). RhoA activation in neutrophils and dHL60 cells was robust after 1 min of UDP-glucose addition, and time course measurements in dHL60 cells indicated that this response persisted for up to 15 min and faded after 30 min. These observations are consistent with studies indicating that rapid, transient increase of RhoA activity in the first minutes after agonist treatment is necessary for migration of leukocytes (1, 55) and other cells (19, 50, 56). They are also in agreement with the notion that RhoA activity is subjected to negative feedback by Rho-GTPase activating proteins (GAPs) and Rho GDP dissociation inhibitor (RhoGDI) (13, 48). Whether additional signaling pathways associated with chemoattractant-evoked responses, e.g., Rac1, Rac2, and Cdc42 (34, 39, 40), contribute to $P2Y_{14}$-R-regulated neutrophil shape change and locomotion remain to be investigated.

ATP release from stimulated phagocytes provides autocrine regulation of directional migration (12). For example, formyl peptides, interleukin 8, C5a complement, and leukotriene LTB4 promote neutrophil migration, which was accompanied by ATP release and inhibited by apyrase or $P2Y_2$-R antagonists (8, 9). In line with these reports, UDP-glucose evoked chemotaxis of neutrophils and dHL60 cells was partially reduced when apyrase was included in the upper level of the Boyden chamber (Figs. 3*B* and 7*B*). Thus, endogenous ATP likely contributed, at least in part, to UDP-glucose-evoked neutrophil chemotaxis.

Recent data from our group demonstrated that, in addition to UDP-sugars, the $P2Y_{14}$-R is activated by UDP (53). Since UDP is also a potent agonist at the $P2Y_6$-R and $P2Y_6$-R transcripts are endogenously expressed in neutrophils and HL60 cells (22), the relative contribution of $P2Y_{14}$-R to UDP-promoted signaling in these cells could not be readily assessed. The effect of UDP on $P2Y_{14}$-R-mediated responses was not investigated in the present study.

Our findings differ from those previously published by Arase et al. (2). These authors reported that 1 μM UDP-glucose had no direct effect on PMN chemotaxis, assessed via the classic transwell filter staining method (2). Addition of 1 μM UDP-glucose to the lower compartment of the chemotaxis chamber in our studies resulted in a relatively minor response, as compared with the response observed with 10 μM UDP-glucose (Fig. 3*C*). However, measurement of UDP-[^{3}H]glucose diffusion through the filter membrane indicated that the concentration of UDP-glucose in the upper compartment of the chamber at the time of quantification of chemotaxis (1 h) was only 20% of the nominal concentration of agonist added to the lower chamber (Fig. 3*C*, *inset*). We suggest that the sensitive quantification provided by the automated, fluorescence-based chemotaxis assay used here as well as the difficulty of accurate estimation of the effective agonist concentration in situ account for the apparent discrepancy between the two studies.

The concept that the $P2Y_{14}$-R plays (patho)physiologically relevant roles is supported by studies demonstrating that UDP-glucose, UDP-galactose, and UDP-*N*-acetylglucosamine are released from cells in a regulated manner (14, 23, 24, 26, 31, 41, 46). Moreover, unlike other proinflammatory molecules that require activation of biosynthetic pathways before their release from cells, UDP-glucose is highly abundant both in the cytosol and secretory pathway of most cells, and it is poised for rapid release together with other nucleotides and nucleotide-sugars from activated and/or damaged cells (30). Importantly, in contrast to short-lived ATP/UTP find-me signals released during sterile inflammatory processes (30, 35, 36), extracellular UDP-glucose is highly stable (31) and is not degraded by neutrophil ecto-nucleotidases (Fig. 1). Thus, UDP-glucose likely is a novel, important local contributor to sterile neutrophilic inflammation.

Our present findings are particularly relevant to human airway pathophysiology. That is, we recently illustrated that Ca^{2+}-regulated exocytosis of specialized mucin granules from goblet cell-reach airway epithelia is accompanied by robust release of UDP-glucose (23, 41). We also have shown that lung secretions from cystic fibrosis patients displaying neutrophil lung inflammation contain elevated levels of UDP-glucose and UDP-*N*-acetylglucosamine, i.e., capable of promoting $P2Y_{14}$-R activation (46). An indirect proinflammatory action of UDP-glucose in the lung has been previously suggested by studies illustrating that UDP-glucose promoted secretion of IL-8 from primary human alveolar type II cell-enriched cultures (38). Our present study suggests an additional, direct role of the neutrophil $P2Y_{14}$-R as a contributor to lung inflammation in chronic lung diseases.

In summary, we demonstrate that UDP-glucose is a highly stable proinflammatory mediator that promotes $P2Y_{14}$-R-regulated RhoA activation, cytoskeleton rearrangements, and chemotaxis in human neutrophils.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.I.S., S.M.K., N.S.-B., R.G.-M., T.K.H., and E.R.L. conception and design of the research; J.I.S., S.M.K., N.S.-B., and E.R.L. performed the experiments; J.I.S., S.M.K., N.S.-B., H.D., R.G.-M., and E.R.L. analyzed the data; J.I.S., S.M.K., N.S.-B., R.G.-M., T.K.H., and E.R.L. interpreted the results of the experiments; J.I.S. and S.M.K. prepared the figures; J.I.S., S.M.K., T.K.H., and E.R.L. approved the final version of the manuscript; T.K.H. and E.R.L. drafted the manuscript; T.K.H. and E.R.L. edited and revised the manuscript.

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