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BRIEF COMMUNICATION

# The inflammatory effects of UDP-glucose in N9 microglia are not mediated by P2Y14 receptor activation

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**Abstract** In this study we evaluated the functionality and inflammatory effects of P2Y14 receptors in murine N9 microglia. The selective P2Y14 receptor agonist UDP-glucose (UDPG) derived from microbial sources dose dependently stimulated expression of cyclooxygenase-2 and inducible nitric oxide synthase, and potentiated the effects of bacterial lipopolysaccharide on nitric oxide production. However, another selective P2Y14 receptor agonist, UDP-galactose, did not affect these endpoints either alone or in combination with lipopolysaccharide. Interestingly, synthetic UDPG also had no detectable pro-inflammatory effects, although P2Y14 receptors are both expressed and functional in N9 microglia. While synthetic UDPG decreased levels of phosphorylated cyclic AMP response element binding protein, an effect that was blocked by pertussis toxin, the pro-inflammatory effects of microbial-derived UDPG were insensitive to pertussis toxin. These data suggest that the pro-inflammatory effects of microbial-derived UDPG are independent of P2Y14 receptors and imply that microbial-derived contaminants in the UDPG preparation may be involved in the observed inflammatory effects.

**Keywords** Cyclic AMP response element binding protein (CREB) · Cyclooxygenase-2 (COX-2) · Inducible nitric oxide synthase (iNOS) · Microglia · Pertussis toxin (PTX) · P2Y14 · UDP galactose

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## Introduction

P2 purinergic receptors potently modulate microglial inflammatory activities, but little is known concerning the effects of P2Y14 receptors in these CNS-resident innate immune cells. Previously, we and others have reported that adenine di- and triphosphate nucleotides, the endogenous ligands for P2 receptors, exert anti-inflammatory effects in microglia [3, 4]; however, the specific P2 receptors involved in these actions are not yet clear. The P2Y14 receptor, previously called GPR105 (an orphan G-protein coupled receptor), has only recently been identified as a member of the purinergic receptor family [1]; hence, comparatively little is known about its function in any cell type. Its newly identified presence in microglia [5] suggests that P2Y14 may have a role in modulating microglial responses in the CNS. Uracil-containing sugar nucleotides, such as uridine 5' diphosphoglucose (UDPG) and UDP-galactose (UDP-gal), are selective agonists for the P2Y14 receptor [1], which couples intracellularly to the inhibition of adenylyl cyclase via Gi/o [1, 8, 10, 11] in many cell types. Adenylyl cyclase catalyzes the formation of cyclic AMP, enabling protein kinase A (PKA) to phosphorylate and

activate the transcription factor cAMP response element binding protein (CREB), among many others. We undertook the present studies to ascertain the role of P2Y14 receptors in modulating microglial cell inflammatory functions.

## Materials and methods

*Materials* The following materials were purchased from Sigma Chemical (St. Louis, MO): LPS (*E.coli* 0111:B4), ATP, UDP-glucose (microbial-derived; catalog #U4625, multiple vials of lot #074K7024 over a period of 15 months) and UDP-galactose (catalog #U4500). Synthetic UDP-glucose was purchased from MP Biomedicals (Solon, OH) and *B. pertussis* toxin (PTX) was obtained from Calbiochem (San Diego, CA).

*Cell culture* Murine N9 microglia [9] were cultured routinely in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro, Herndon, VA) supplemented with 5% fetal bovine serum (Bio Whittaker, Walkersville, MD) and 100 U/ml penicillin/streptomycin (Cellgro) in 100-mm Sarstedt plates. Cells were grown to ~90% confluency and passaged every 2 days. For experimentation, cells were plated at densities of $1\times10^5$ cells per well in 24-well plates for nitrite measurements, or at $4\times10^5$ cells per well in 12-well plates for signaling studies and RT-PCR. The next day, the cells were treated as specified below.

*Reverse-transcription polymerase chain reaction (RT-PCR)* RT-PCR was performed on 1 μg of total RNA from whole brain (positive control) and N9 microglia as previously reported [2, 4]. RT reactions were completed according to the manufacturer's protocol with and without reverse transcriptase (+/-RT). The cDNA was then used for PCR using the GoTaq Green Master Mix (Promega, Madison, WI). The murine P2Y14 receptor gene was amplified using the following primers: 5'TAGAGGCCATAAACTGTGC TT and 5'AATTCTTCCTGGACTTGAGGT (expected amplimer size 742 bp). As a control, GAPDH (glyceraldehyde-3-phosphate dehydrogenase; expected amplimer size 325 bp) was amplified using the following primers: 5'CCAT CACCATCTTCCAGGAG and 5'GATGGCATG GACTGTGGTC. PCR products were separated and visualized by ethidium bromide-stained 1% agarose gel electrophoresis.

*Measurement of nitrite production* Cells were stimulated with LPS (1 μg/mL) either alone or together with UDPG for 18–22 h at the concentrations indicated. In some experiments, cells were treated with increasing concentrations of UDPG or UDP-gal alone (50–300 μM), or with UDPG that had been boiled at 105°C for 30–60 min. In other experiments, microglia were pretreated with PTX (100 ng/mL) for 18 h, followed by stimulation with UDPG. Nitric oxide (NO) levels were measured in the culture medium using the Greiss reagent to analyze nitrite production, a stable breakdown product of NO generation [7]. The cells were collected for immunoblot analyses.

*Immunoblot analyses* Whole-cell lysates were prepared and total protein content determined as previously described [4]. Equal amounts of protein (~25 μg) were loaded per lane and separated by 10% SDS-PAGE [6]. Proteins in the gels were transferred to Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), and the membranes were subjected to immunoblot analyses for iNOS, COX-2 and phospho-CREB as described previously

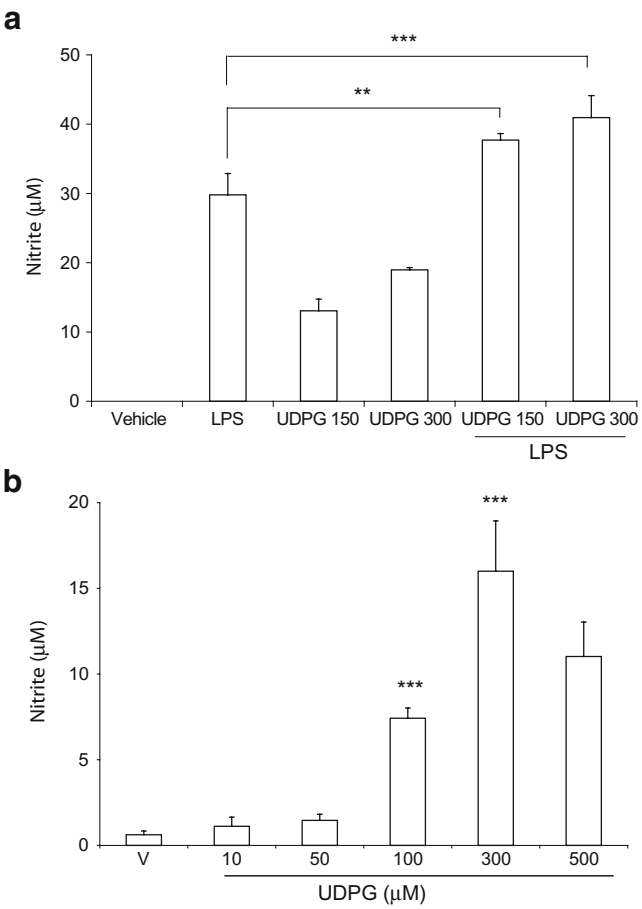


**Fig. 1** UDPG potentiates LPS-stimulated NO release from N9 microglia. **a** Microglia were stimulated with vehicle (250 mM Hepes), LPS (1 μg/ml), UDPG (150 and 300 μM), or LPS and UDPG together for 18–22 h. Nitrite levels were determined using the Griess reagent. The graph is representative of at least five independent experiments performed in triplicate. **b** Microglia were treated with microbial-derived UDPG at the indicated concentrations for 18–22 h and nitrite levels assessed. $^{**}P<0.01$, $^{***}P<0.001$

[4]. To confirm equal protein loading, membranes were probed with antibodies recognizing the cytosolic proteins Grb-2 or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). The data shown are representative of at least three independent experiments performed in triplicate.

*Statistical analyses* Statistical analyses were performed using an ANOVA pre-hoc test and Tukey-Kramer, Dunnett or Bonferroni multiple comparison post-hoc analyses. Statistical significance was set at the 95% confidence limit ($P<0.05$). Quantitative data are expressed as the mean ± SD of three to six independent experiments.

## Results

### UDP-glucose stimulates nitric oxide (NO) production in N9 microglia

To investigate the role of P2Y14-receptor activation in microglia, we stimulated N9 cells with vehicle (Hepes buffer), LPS (1 μg/mL), the P2Y14 agonist UDPG (150 or 300 μM), or combinations of both LPS and UDPG. LPS strongly induced nitrite accumulation that was further increased in the presence of UDPG (Fig. 1a), indicating that microglia are more activated in the presence of LPS

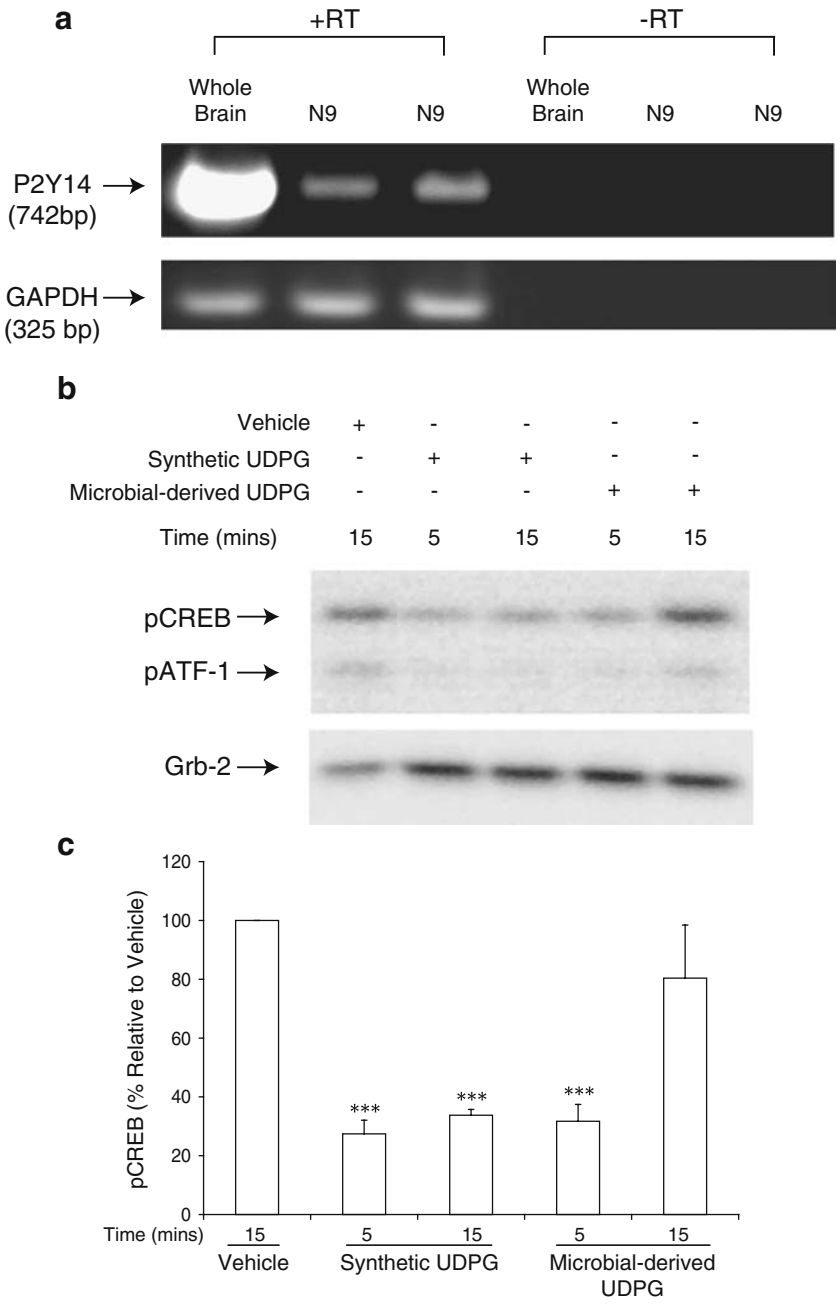


**Fig. 2** P2Y14 receptors are expressed and functional in N9 microglia. **a** Total RNA was isolated from two independently cultured N9 microglial thaws and from mouse whole brain as a positive control. The reverse transcription reaction was performed with (+) or without (−) reverse transcriptase (RT) followed by PCR amplification of P2Y14 mRNA (*upper panel*) or GAPDH mRNA (*lower panel*) as a control. **b** Microglia were stimulated in triplicate for 5 or 15 min with vehicle (250 mM Hepes), synthetic UDPG (300 μM), or microbial-derived UDPG (300 μM). Proteins from triplicate treatments were pooled and immunoblot analyses performed for phosphorylated CREB. Membranes were re-probed for the presence of Grb-2 as a loading control. The figure is representative of at least three independent experiments each performed in triplicate. **c** Quantification of phosphorylated CREB (pCREB) immunoreactivity obtained from the densitometric analyses of immunoblot studies ($n\geq3$) performed as shown in **b**. Normalized values (using Grb-2 immunoreactivity as a loading control) are expressed as percent CREB phosphorylation relative to vehicle treatment, and the data represent the mean ± SD of at least three separate experiments. $^{**}P<0.01$ vs. vehicle

and UDPG together. Maximum levels of UDPG-stimulated nitrite accumulation occur between 100 and 300 μM UDPG (Fig. 1b); therefore, 300 μM UDPG concentrations were used in subsequent experiments. Because UDPG on its own can robustly increase NO production, activation of P2Y14 receptors may be a pro-inflammatory signal for microglia.

## P2Y14 receptors are functionally expressed in N9 microglia

To discern the specificity of the UDPG response, we evaluated the expression of P2Y14 receptors in N9 microglia by RT-PCR. Although not as great as in whole brain, P2Y14 receptor mRNA was detectably expressed in N9 microglia (Fig. 2a). Because P2Y14 receptors couple to Gi, we tested the ability of P2Y14-receptor ligands to decrease CREB phosphorylation. As we have reported previously, basal levels of phosphorylated CREB (pCREB) are relatively high in microglia [4]. We therefore evaluated the ability of synthetic UDPG (300 μM) and microbial-derived UDPG (300 μM) to decrease CREB phosphorylation as an indicator of their ability to functionally couple to the Gi/adenylyl cyclase/cAMP/PKA pathway. Synthetic UDPG decreased basal CREB phosphorylation levels at both 5 and 15 min, whereas microbial-derived UDPG did so only at the earlier time point (Fig. 2b,c). Another P2Y14-receptor agonist, UDP-gal (300 μM), also decreased CREB phosphorylation at both intervals (data not shown).

P2Y14 receptor-mediated reductions in CREB phosphorylation were evident at synthetic UDPG concentrations between 10 and 50 μM, and they appeared to plateau between 10 and 300 μM synthetic UDPG (Fig. 3a). Importantly, these decreases in CREB phosphorylation were prevented in the presence of PTX (Fig. 3b), suggesting the involvement of P2Y14 receptors and Gi/o heterotrimeric G proteins in these effects.

## Microbial-derived UDPG-stimulated NO production is insensitive to pertussis toxin

To ascertain if the pro-inflammatory effects of microbial-derived UDPG in N9 microglia were mediated by activation of P2Y14 receptors, we used PTX to prevent Gi/o proteins from interacting with the P2Y14 receptor. As

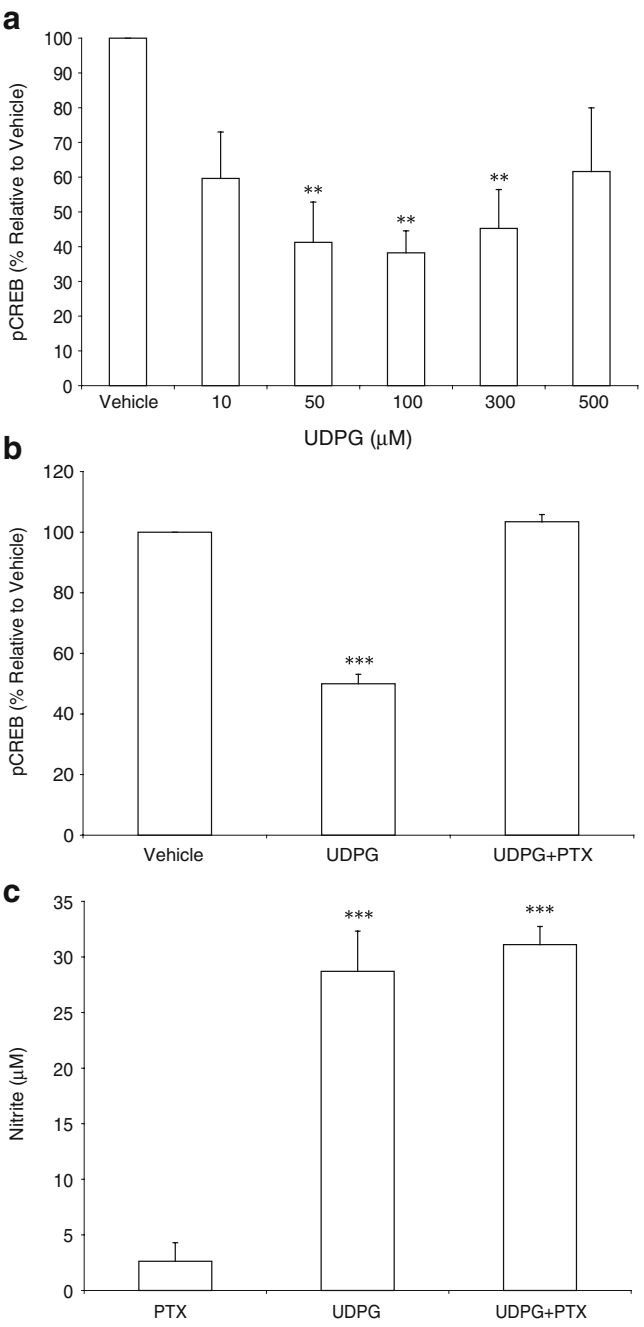


**Fig. 3** Reductions in CREB phosphorylation stimulated by synthetic UDPG are sensitive to PTX, whereas microbial-derived UDPG-stimulated NO production is PTX insensitive. Quantification of phosphorylated CREB immunoreactivity obtained from the densitometric analyses of immunoblot studies ($n \geq 3$). **a** Microglia were stimulated in triplicate with synthetic UDPG at the concentrations indicated for 15 min. **b** Microglia were pretreated with pertussis toxin (PTX; 100 ng/mL) for 18 h prior to stimulation with synthetic UDPG for 15 min. Proteins from triplicate treatments were pooled and immunoblot analyses performed for phosphorylated CREB. Membranes were re-probed for the presence of Grb-2 as a loading control. Normalized numerical values (using Grb-2 immunoreactivity as a loading control) are expressed as percent CREB phosphorylation relative to vehicle treatment, and the data represent the mean ± SD of at least three separate experiments. $^{**}P<0.01$ and $^{***}P<0.001$ vs. vehicle. **c** Microglia were pretreated in triplicate with vehicle (PBS) or PTX (100 ng/mL) for 18 h prior to stimulation with either vehicle (250 mM Hepes) or UDPG (300 μM) for 18–22 h. Nitrite levels were determined using the Griess reagent. The graph represents cumulative quantification of at least three independent experiments, wherein treatments were performed in triplicate. $^{***}P<0.001$ vs. PTX alone

shown in Fig. 3c, the presence of PTX did not interfere with the ability of UDPG to stimulate nitrite accumulation, suggesting a P2Y14 receptor-independent effect of microbial-derived UDPG.

## UDP-galactose is without effect on inflammatory gene expression in N9 microglia

To further examine a role for P2Y14 receptors in promoting the pro-inflammatory effects of microbial-derived UDPG, we used another P2Y14 receptor agonist, UDP-gal. Unlike in the case of UDPG, we did not observe any effect of UDP-gal (75–300 μM) on either NO production (Fig. 4a) or on the expression of inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2; Fig. 4b). We have used concentrations of UDP-gal as high as 5 mM and found no detectable pro-inflammatory effects in our cell line (data not shown). If P2Y14 receptors mediated the responses to UDPG, then UDP-gal would be expected to act similarly. Interestingly, boiled UDPG retained the ability to dose dependently increase NO production (Fig. 4a), supporting the idea that the UDPG contaminant is not heat labile, consistent with the presence of microbial cell wall products.

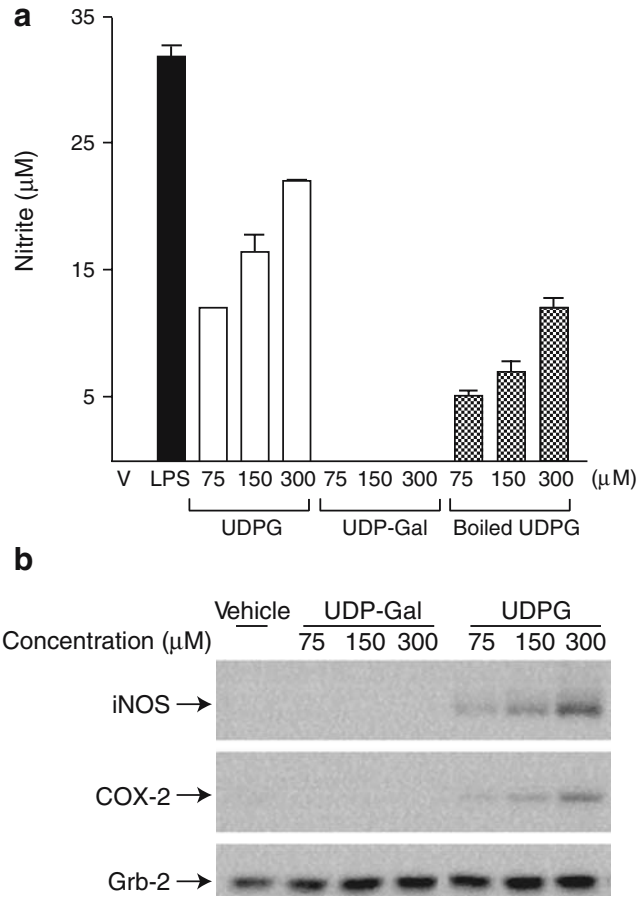


**Fig. 4** The P2Y14-receptor ligand UDP-glucose, but not UDP-galactose, is pro-inflammatory in N9 microglia. Microglia were stimulated in triplicate with vehicle (V; 250 mM Hepes), UDPG (75, 150, or 300 μM), UDP-gal (75, 150, or 300 μM), or boiled UDPG (75, 150, or 300 μM) for 18–22 h. **a** Nitrite levels in the medium were determined using the Griess reagent. **b** Proteins were harvested, pooled and analyzed by immunoblot analyses for iNOS (*upper panel*) and COX-2 (*middle panel*). Grb-2 was used to control for protein loading (*lower panel*). The data shown are representative of at least three independent experiments

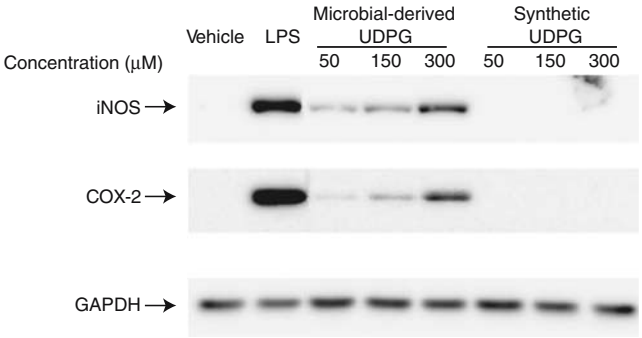


**Fig. 5** Microbial-derived UDPG stimulates iNOS and COX-2, whereas synthetic UDPG does not. Microglia were stimulated in triplicate for 18–22 h with either vehicle (250 mM Hepes), synthetic UDPG (50, 150, or 300 μM) or microbial-derived UDPG (50, 150, or 300 μM). Proteins from triplicate treatments were pooled and immunoblot analyses were performed for iNOS and COX-2 protein levels. Membranes were re-probed for the presence of GAPDH as a loading control. The figure is representative of at least three independent experiments performed in triplicate

## Synthetic UDP-glucose lacks pro-inflammatory activities in N9 microglia

The microbial-derived UDPG that yielded the observed pro-inflammatory effects in these studies was purchased from a company that supplies the UDPG used in more than 90% of publications on P2Y14 receptors. Only a single lot number (although multiple vials) of this UDPG (derived from the microbial source *Saccharomyces cerevisiae)* was available over the course of the 15-month duration of these experiments. Because our data indicated that the pro-inflammatory activity in the microbial-derived UDPG preparation is independent of P2Y14 receptors, we purchased a chemically synthesized UDPG preparation from a different company (synthetic UDPG). As shown in Fig. 5, synthetic UDPG was without effect on iNOS and COX-2 expression in N9 microglia, although it was capable of reducing basal CREB phosphorylation levels (Fig. 3a).

## Discussion

These studies indicate that UDPG preparations derived from microbial sources may have pro-inflammatory activ-

ities that are independent of P2Y14 receptor activation. In addition, our data suggest that selective activation of P2Y14 receptors (by synthetic UDPG or by UDP-gal) has no effect on the production of the inflammatory mediators iNOS/NO and COX-2 by N9 microglia. Because synthetic UDPG and UDP-gal inhibit the phosphorylation of CREB in a manner that is sensitive to pertussis toxin, P2Y14 receptors appear to be expressed and functional in N9 microglia. However, because the pro-inflammatory effects of microbial-derived UDPG on NO production were not blocked by PTX, it is likely that these stimulatory effects in microglia do not involve P2Y14 receptors.

Although the function of P2Y14 in any cell type remains unclear, in immature dendritic cells, activation of P2Y14 receptors increases expression of CD86, suggesting their involvement in dendritic cell maturation [13]. Also, pro-inflammatory stimuli such as LPS [8] and interferon-γ (JMC and JJW, unpublished observations) increase the expression levels of GPR105 in glial cells and in N9 microglia, respectively, supporting the idea that this receptor plays a role in the microglial response to pro-inflammatory stimuli. While we did not observe any modulatory effects of selective P2Y14 receptor activation on iNOS or COX-2 pathways in microglia, it remains probable that P2Y14 receptors alter some other microglial inflammatory function(s) not measured here.

It is important to note that in addition to our study, other literature reports also indicate different responses to UDPG and UDP-gal. Whereas both UDPG and UDP-gal are agonists of P2Y14 receptors [1, 14], in human neutrophils [12] and in murine T-lymphocytes [10] UDP-gal does not mimic the effects of UDPG. Coincidentally, the source of UDPG used in the above studies was also microbial-derived. In a separate set of studies, microbial-derived UDPG had effects in a cell line in which P2Y14 receptors were not detectably expressed [11], supporting the idea that UDPG preparations from microbial sources may contain some other activity that is independent of P2Y14 receptors. Because many studies on P2Y14 receptor function are performed in transfected cell systems, potential microbial contaminants in the nucleotide preparations are admittedly not as large a concern as when studies are performed in immune cells, which can strongly respond to microbial products. However, based on the data presented here, the presence of microbial products in the nucleotide preparation may significantly alter data interpretation.

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