Kynurenic Acid as a Ligand for Orphan G Protein-coupled Receptor GPR35*

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Local catabolism of the essential amino acid tryptophan is considered an important mechanism in regulating immunological and neurological responses. The kynurenine pathway is the main route for the non-protein metabolism of tryptophan. The intermediates of the kynurenine pathway are present at micromolar concentrations in blood and are regulated by inflammatory stimuli. Here we show that GPR35, a previously orphan G protein-coupled receptor, functions as a receptor for the kynurenine pathway intermediate kynurenic acid. Kynurenic acid elicits calcium mobilization and inositol phosphate production in a GPR35-dependent manner in the presence of Gqoi chimeric G proteins. Kynurenic acid stimulates [35S]guanosine 5'-O-(3-thiotriphosphate) binding in GPR35-expressing cells, an effect abolished by pertussis toxin treatment. Kynurenic acid also induces the internalization of GPR35.

**EXPERIMENTAL PROCEDURES**

**Cloning and Cell Culture**—Full-length human, mouse, and rat GPR35 were cloned by PCR from human universal cDNA, mouse spleen cDNA, and rat spleen cDNA (BD Bioscience Clontech), respectively. Sequence-confirmed cDNAs were inserted into the mammalian expression vector pcDNA3.1 (Invitrogen). Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Cellgro) containing 10% fetal bovine serum and antibiotics. HeLa and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. All cell lines were cultured at 37°C with 5% CO2. CHO-GPR35 stable cells were generated by transfecting CHO cells with N-terminal-FLAG-tagged human GPR35 and subsequently selected in 1 mg/ml G418 (Invitrogen). Flow cytometry analysis was carried out on FACSCalibur (BD Biosciences) after staining with anti-FLAG M2 monoclonal antibody (Sigma) and goat anti-mouse IgG-fluorescein isothiocyanate secondary antibody (Caltag). All compounds tested were from Sigma.

**Aequorin Assay**—CHO cells were transfected with either empty vector or vector expressing GPR35 together with the aequorin reporter plasmid using Lipofectamine 2000 reagent (Invitrogen) (5, 19). For each 10-cm dish, 5 μg of GPR35 and 5 μg of aequorin reporter plasmids were used. When indicated, 2 μg of plasmids expressing small G proteins (Gα16, Gqα5, Gqα9, and/or Gqα11) (20–23) were also included. 24 h after transfection...
cells were harvested and resuspended in Hanks’ buffered salt solution containing 0.01% bovine serum albumin and 20 mM HEPES (Cellgro), loaded with 1 μM coelenterazine f (P. J. K. Industrievertretungen, Handel, Germany) at room temperature for 1 h, and stimulated with compounds. Ligand-induced calcium mobilization, as indicated by an increase in aequorin luminescence, was recorded over a period of 20 s with a Microlumat luminometer (Berthold).

### Inositol Phosphate Accumulation Assay
HEK293 cells seeded in 96-well plates were transfected with GPR35 (100 ng/well) and small G proteins (Gα16, Gqα5, Gqα9, and Gqα5). After labeling with [3H]myoinositol (Amersham Biosciences) for 16 h, cells were stimulated with compounds in Hanks’ buffered salt solution, 25 mM Hepes (pH 7.4), 10 mM LiCl, 0.01% bovine serum albumin at 37 °C for 1 h. 20 mM formic acid was used to lyse the cells at 4 °C for 4 h. Ysi-SPA beads (Amersham Biosciences) were added to the cell lysates and incubated overnight in the dark. Radioactivity was recorded on a Topcount 96/384 scintillation counter (Packard).

### GTPγS Binding Assay
CHO-GPR35 stable cells were pre-treated with or without pertussis toxin (Calbiochem, 100 ng/ml) for 16 h before harvesting. Cells were resuspended and homogenized in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA followed by centrifugation at 1000 × g for 10 min at 4 °C to remove nuclei and cellular debris. Membrane fractions were collected by spinning the supernatant at 38,000 × g for 30 min and resuspended in 20 mM HEPES (pH 7.5) and 5 mM MgCl2. 25 μg of membranes was incubated at room temperature for 1 h in assay buffer (20 mM HEPES, 5 mM MgCl2, 0.1% bovine serum albumin (pH 7.5)) containing 3 μM GDP and 0.1 nM [35S]GTPγS (PerkinElmer Life Sciences) in the absence or presence of kynurenic acid. Reactions were terminated by vacuum filtration through GF/B filters, and the retained radioactivities were quantified on liquid scintillation counter.

### Immunofluorescence Staining
HeLa cells were seeded on coverslips in 6-well plates and transfected with N-terminal-

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**FIGURE 1. Identification of kynurenic acid as a ligand for GPR35.** A, dose-dependent activation of GPR35 by kynurenic acid. CHO cells were transfected with human GPR35 or vector control in the presence of plasmids expressing aequorin reporter and G protein mixture (Gα16, Gqα5, Gqα9, and Gqα5). Ligand-induced [Ca²⁺], increase was recorded as aequorin luminescence signal. RLU, relative luminescence units. Data shown are the means ± S.E for duplicate determinations. B, structure of kynurenic acid. C, tryptophan metabolic pathway (18). The kynurenine pathway, the main route for the metabolism of tryptophan, is shown in bold.

**FIGURE 2. Activation of GPR35 from various species by kynurenic acid.** Human (A), mouse (B), or rat GPR35 (C) was tested in aequorin assay in the presence of a co-transfected G protein mixture (Gα16, Gqα5, Gqα9, and Gqα5). [Ca²⁺], increase, shown as aequorin luminescence signal, was recorded. Data are shown as the means ± S.E for duplicate determinations. Quinolinic acid, another metabolite in tryptophan pathway, did not activate GPR35. RLU, relative luminescence units.

### TABLE 1

| Tryptophan metabolites potency in aequorin assay | Human GPR35 | Mouse GPR35 | Rat GPR35 |
|-----------------------------------------------|------------|------------|-----------|
| EC₅₀ in μM                                      |            |            |           |
| Kynurenic acid                                 | 39.2       | 10.7       | 7.4       |
| Quinolinic acid                                | Inactive   | Inactive   | Inactive  |
| Kynurenine                                     | >1000      | >1000      | >1000     |
| Anthranilic acid                               | Inactive   | Inactive   | Inactive  |
| 3-Hydroxykynurenine                            | Inactive   | Inactive   | Inactive  |
| 3-Hydroxyanthranilic acid                      | Inactive   | Inactive   | Inactive  |
| Picolinic acid                                 | Inactive   | Inactive   | Inactive  |
| Pyridoxaldehyde                                | Inactive   | Inactive   | Inactive  |
| Tryptophan                                     | Inactive   | Inactive   | Inactive  |
| Xanthurenic acid                               | Inactive   | Inactive   | Inactive  |
| Serotonin                                      | Inactive   | Inactive   | Inactive  |
| Melatonin                                      | Inactive   | Inactive   | Inactive  |
FLAG-tagged human, mouse, or rat GPR35. For surface staining, cells were fixed with 4% paraformaldehyde, blocked with 5% goat serum in phosphate-buffered saline (Cellgro), incubated with anti-FLAG M1 monoclonal antibody (Sigma) for 1 hour, and then washed extensively in phosphate-buffered saline before incubating with goat anti-mouse IgG-rhodamine secondary antibody for an additional 30 minutes. Internalization of GPR35 was induced by kynurenic acid (300 μM) for 30 minutes at 37 °C. After ligand stimulation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton before staining with antibodies. Images were captured with a CCD digital camera connected to a Leica DC500 microscope.

Quantitative RT-PCR Analysis—Total RNA from human or mouse tissues (BD Biosciences Clontech) were treated with DNase I (Ambion) before reverse transcription. Quantitative reverse transcriptase-PCR was performed on an ABI Prism 7700 sequence detector using Taqman PCR core reagents (Applied Biosystems). Ratios of GPR35 to glyceraldehyde-3-phosphate dehydrogenase message RNA were calculated using a ΔΔCt method (Applied Biosystems). Primers and probes were designed using Primer Express software (Applied Biosystems). Primer and probe sequences for human GPR35 were GTGCCCTCCTGGAGACGAT (forward) and GCAGCAGTTGGCATCTGAGA (reverse) and for the probe, 5'-FAM-CGTCGCGCCCTGTA-CATAACCAGC-BHQ-3'. (FAM, 6-carboxyfluorescein; BHQ, black hole quencher). Primer and probe sequences for mouse GPR35 were ATCACAGGTAAACTCTCAGACACCAACT (forward) and CTTGAACGCTTCCTGGAACTCT (reverse) and for the probe, 5'-FAM-TGGATGCCATCTGTTACTACTACT-TGGCCA-BHQ-3'.

In Situ Hybridization—Mouse GPR35 cDNA cloned in pCMV-SPORT6 vector (Invitrogen) was used as a template for generating RNA probes using T7 and SP6 RNA polymerase (Promega). [33P]UTP-labeled antisense or sense GPR35 RNA probes were hybridized to paraformaldehyde-fixed, paraffin-embedded mouse tissue array (Imgenex). Hybridization buffer contained 50% formamide, 300 mM NaCl, 20 mM Tris-Cl
(pH 8.0), 10 mM NaH₂PO₄, 10% dextran sulfate, 1/₁₁₀₀₀₀ Denhardt’s solution, 0.5 mg/ml tRNA as described (24). After extensive washing, slides were exposed to x-ray film, dipped in emulsion type NTB (Kodak), and developed after 3 weeks. Sections were counterstained with hematoxylin for nuclear visualization.

**Cytokine Secretion**—Human peripheral blood mononuclear cells and CD₁₄⁺ monocytes were purchased from Allcells. Peripheral blood mononuclear cells were seeded at a density of 2 × ₁₀⁶ cells/ml, and monocytes were seeded at 2 × ₁₀⁵ cells/ml in 24-well plates in RPMI 1640 medium. Kynurenic acid was added 1 h before lipopolysaccharides (LPS (Sigma), final concentration at 10 ng/ml). Cells were incubated at 37 °C for 18 h, and supernatant was collected for cytokine assay. Untreated cells were used as controls. TNF-α concentrations were determined with Quantikine enzyme-linked immunosorbent assay kits (R&D Systems) following the manufacturer’s instructions.

**RESULTS**

To search for natural ligands for orphan GPCRs, we tested a collection of ~300 biochemical intermediates for their ability to evoke an increase in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) using the aequorin assay (19). CHO cells were transiently transfected with plasmids encoding human GPR35, aequorin reporter, and a mixture of promiscuous or chimeric small G proteins (Gₐ₁₆, Gₐ₉₅, Gₐ₉ₒ⁵, and Gₐ₉ₒ), which have been reported to couple with GPCRs that are not normally linked to Ca²⁺ signaling and shift their signal transduction to calcium mobilization (21–23). Kynurenic acid evoked a specific rise in [Ca²⁺]ᵢ in cells expressing human GPR35 and G protein mixture, with a medium effective concentration (EC₅₀) of 3.₉ M (Fig. 1A). No specific response was observed in control cells (Fig. 1A). Kynurenic acid (structure shown in Fig. 1B) is a metabolite in the tryptophan metabolic pathway (Fig. 1C).

Kynurenic acid also activated mouse and rat orthologues of GPR35 (Fig. 2). Quinolinic acid, another metabolite of the tryptophan pathway and often indicated to have opposing functions to kynurenic acid (16), produced no response (Fig. 2). Interestingly, kynurenic acid is more potent on rodent GPR35 than on human GPR35, with EC₅₀ values of 1.₁ and 7 M for mouse and rat GPR35, respectively. GPR35 was selectively activated by kynurenic acid but not by other tryptophan metabolic pathway intermediates (Table 1) such as 3- hydroxyanthranilic acid and 3-hydroxykynurenine that are implicated in T cell regulation (25). Of ~300 biochemical intermediates, kynurenic acid was found to be the most potent in stimulating GPR35 (data not shown). Furthermore, kynurenic acid did not activate ~40 other GPCRs, including GPR55, the closest homologue of GPR35 (data not shown).

To dissect the signaling pathways of GPR35, CHO cells were transfected with plasmids encoding human GPR35 and individual small G proteins and tested in aequorin assay. The Gₐₒ/ᵩ chimeras (22), Gₐ₉ₒ⁵ and Gₐ₉ₒ⁵, significantly potentiated the activation of GPR35 by kynurenic acid, whereas Gₐₒ chimera (Gₐ₉ₒ) and the promiscuous G protein Gₐₒ₋ₑ did not (Fig. 3). The use of Gₐₒ/ᵩ chimera was reported to allow the Gₐₒ-coupled GPCRs to signal via the Gq pathway, leading to calcium mobilization (21).
These results suggest that GPR35 may signal through Gi/o pathways in CHO cells.

Kynurenic acid induced the accumulation of inositol phosphate in HEK293 cells transiently transfected with GPR35 and Gq/5 (Fig. 4). No inositol phosphate formation was detected in the absence of co-transfected G proteins, suggesting that GPR35 may not signal through the Gq pathway (Fig. 4). These results agree with the observation that the Gqi/o chimeras significantly enhanced GPR35 activation in the aequorin assay (Fig. 3).

Flow cytometry analysis showed surface expression of GPR35 on CHO cells stably expressing N-terminal-FLAG-
tagged human GPR35 but not vector control cells (Fig. 5A). Kynurenic acid stimulated \([^{35}S]GTP_S\) incorporation in membrane preparations from CHO-GPR35 cells, an effect abolished by preincubation with pertussis toxin (Fig. 5B). CHO-vector control cells did not respond to kynurenic acid (data not shown). The EC50 for kynurenic acid-induced activation of human GPR35 in \([^{35}S]GTP_S\) binding assay was 36 nM, similar to the EC50 value obtained from the aequorin assay. These results, together with the preference for Gqi/o chimeras in the aequorin and inositol phosphate formation assays suggest that GPR35 activation by kynurenic acid couples to a pertussis toxin-sensitive Gi/o pathway.

Ligand-induced receptor internalization is often characteristic of GPCR activation and signal attenuation (26). Immunofluorescence staining of cells expressing N-terminal-FLAG-tagged human, mouse, or rat GPR35 revealed that GPR35 proteins from different species were localized to the plasma membrane (Fig. 6A). In contrast, a FLAG-tagged protein IKKβ (27) exhibited an expected intracellular localization (Fig. 6A). Kynurenic acid stimulation induced the translocation of GPR35 from plasma membrane to punctate intracellular structures (Fig. 6B), a characteristic of receptor internalization.

Expression analysis by quantitative reverse transcriptase-mediated PCR revealed that both human GPR35 and mouse GPR35 were predominantly expressed in immune and gastrointestinal tissues, with limited expression in other tissues (Fig. 7). In humans, GPR35 messenger RNA was mainly detected in the peripheral leukocytes, spleen, small intestine, colon, and stomach (Fig. 7A). In mice, high levels of GPR35 expression were detected in the spleen and gastrointestinal tract (Fig. 7B). Similar results were obtained using primers and probes annealing to different regions of GPR35 (data not shown). Among various subpopulations of immune cells, GPR35 was detected in CD14+ monocytes, T cells, neutrophils, and dendritic cells, with lower expression in B cells, eosinophils, basophils, and platelets (Fig. 7C).

In situ hybridization experiments using mouse multiple tissue arrays corroborated with quantitative reverse transcriptase-PCR data. Specific GPR35-positive signals were detected in the spleen and gastrointestinal tract, including duodenum, jejunum, ileum, cecum, colon, and rectum (Fig. 8A). GPR35 sense probe did not generate significant signals in these tissues (Fig. 8A). In various regions of the intestine (duodenum in Fig. 8B, ileum in Fig. 8C, and colon in Fig. 8D), GPR35 was
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binding in a GPR35-dependent manner and induced the internalization of GPR35. The discovery of kynurenic acid as an endogenous ligand for GPR35 further highlighted the importance of tryptophan catabolism in regulating biological functions.

The tryptophan metabolic pathway leading to the synthesis of kynurenine (kynurenine pathway) is the main route for non-protein metabolism of the essential amino acid tryptophan. Of the dietary tryptophan intake that is converted biochemically into other compounds, ~99% is metabolized by the kynurenine pathway (16). Given its roles in immunity and the central nervous system, the kynurenine pathway has emerged as an attractive target for drug development (16, 17, 25, 28). As an intermediate of the kynurenine pathway, kynurenic acid has been shown to be a naturally occurring antagonist for NMDA glutamate receptor (IC₅₀ ~ 8–15 μM in the absence of glycine; IC₅₀ ~ 230 μM in the presence of 10 μM glycine) (29, 30) and mediates a neuroprotective effect. Another intermediate in the pathway, quinolinic acid, could excite neurons and cause neurotoxicity in the central nervous system by acting as an agonist at the NMDA receptors (17, 18). The EC₅₀ values of kynurenic acid on GPR35 activation reported in the current study are comparable with the concentration required for NMDA receptor antagonism.

Tryptophan metabolic pathway is activated during inflammatory conditions such as viral invasion, bacterial lipopolysaccharide, or interferon-γ stimulation (31, 32). The activation of tryptophan metabolism causes a reduced plasma tryptophan level and an elevation of kynurenine acid level (16). Kynurenic acid has a basal plasma level of ~140 nM (33), and its concentration is substantially elevated to micromolar level by inflammatory responses and cytokine release (34–37). The concentrations of kynurenic acid required for GPR35 activation in in vitro assays are higher than basal plasma kynurenic acid level (EC₅₀ values were ~39, 11, and 7 μM for human, mouse, and rat GPR35, respectively, in aequorin assays). However, it is frequently observed that a much higher ligand concentration is required when G₁₅₀-coupled receptors are converted to G₅₂ signaling by coexpressing chimeric G proteins (11, 38, 39). Furthermore, serum or plasma measurements only reflect the concentration of kynurenic acid after diffusion and dilution from the site of release and, therefore, may underestimate the effective concentration at the site of action. This is particularly true when a local inflammatory reaction occurs after infection, injury, or immune cell activation. Because kynurenic acid is a metabolite that is not subject to rapid subsequent metabolism, it is conceivable that higher concentrations than blood levels can be achieved in local tissues to evoke a significant biological effect.

The predominant expression of GPR35 in immune cells and the elevation of kynurenic acid levels during inflammation suggest that this receptor-ligand pair may play important roles in immunological regulation. In fact, kynurenic acid inhibited LPS-induced TNFα secretion in peripheral blood monocytes (Fig. 9). Because the tryptophan metabolic pathway is activated by pro-inflammatory stimuli, the anti-inflammatory effect of kynurenic acid provides an interesting feedback mechanism in modulating immune responses. More in depth studies are needed to address whether the anti-inflammatory effects of kynurenic acid are mediated by GPR35 activation.

DISCUSSION

In the current study we have identified kynurenic acid, an intermediate in the tryptophan metabolic pathway, as a ligand for GPR35. Kynurenic acid activated GPR35 in aequorin assay and inositol phosphate accumulation assay in the presence of G₁₅₀ chimeric G proteins. Kynurenic acid also stimulated [³⁵S]GTPγS

primarily expressed in the epithelial cells located in the crypts of Lieberkühn, with lower expression in the intestinal villi. No significant signals were detected in lamina propria, muscularis propria, and enteric neurons (data not shown).

To investigate the potential biological functions of kynurenic acid on immune cells expressing GPR35, we tested the effect of kynurenic acid on cytokine secretion in human peripheral blood mononuclear cells. Kynurenic acid by itself did not stimulate TNFα secretion in these cells (data not shown). However, kynurenic acid was able to attenuate LPS-induced TNFα secretion in a dose-dependent manner (Fig. 9A). Similar results were obtained using purified peripheral blood CD14⁺ monocytes (Fig. 9B).

FIGURE 9. Inhibition of LPS-induced TNF-α release by kynurenic acid. Human peripheral blood mononuclear cells (A) or CD14⁺ monocytes (B) were incubated with kynurenic acid (mM) in the absence or presence of 10 ng/ml LPS. 18 h after the treatment, concentrations of TNFα in cell culture supernatants were determined.
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GPR35 is enriched in the intestinal crypts of Lieberkühn, which are rich in actively proliferating stem cells and progenitor cells crucial for the self-renewal of gastrointestinal epithelium (40). Interestingly, elevated plasma levels of kynurenic acid are reported in patients with inflammatory bowel diseases (36, 37). The involvement of GPR35 in inflammatory bowel diseases such as ulcerative colitis and Crohn disease and other gastrointestinal disorders should be further investigated.

GPR35 has limited expression in the brain, where kynurenic acid is known to exert neuroprotective effects (18). Although mechanisms such as NMDA receptor blockade by kynurenic acid have been recognized, the roles of GPR35 in these processes are unknown. It remains to be investigated whether some of the effects mediated by kynurenic acid in the brain might result from GPR35 activation. GPR35-deficient mice will be valuable for dissecting the biological functions of kynurenic acid.

GPR35 has been reported as a potential oncogene implicated in gastric cancer (41). The kynurenine pathway is activated in immune cells surrounding the core of solid tumors (16). It is possible that kynurenic acid produced by activated immune cells could stimulate the abnormal growth of gastrointestinal cells expressing GPR35, contributing to tumorigenesis in these tissues. Thus, we have identified the tryptophan metabolite kynurenic acid as an endogenous ligand for GPR35. This study together with others (5–10) suggests that metabolic intermediates previously believed to be biologically inactive merit further investigation. The signaling functions of these chemicals shall emerge as a new area for pharmacological studies. The newly identified receptors for these metabolic intermediates shall present novel opportunities for drug discovery and development.

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