Identification of GPR99 Protein as a Potential Third Cysteiny1 Leukotriene Receptor with a Preference for Leukotriene E4 Ligand

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The cysteinyl leukotrienes (cys-LTs), leukotriene C4 (LTC4), a conjugation product of glutathione and eicosatetraenoic acid, and its metabolites, LTD4 and LTE4, are lipid mediators of smooth muscle constriction and inflammation in asthma. LTC4 is the most potent ligand for the type 1 cysteinyl leukotriene receptor (CysLT1R), and LTC4 and LTD4 have similar lesser potency for CysLT2R, whereas LTE4 has little potency for either receptor. CysLT1R/CysLT2R mice, lacking the two defined receptors, exhibited a comparable dose-dependent vascular leak to intradermal injection of LTC4 or LTD4 and an augmented response to LTE4 as compared with WT mice. As LTE4 retains a cysteine residue and might provide recognition via a dicarboxylic acid receptor, we screened cDNAs within the P2Y nucleotide receptor family containing CysLTRs and dicarboxylic acid receptors with trans-activator reporter gene assays. GPR99, previously described as an oxoglutarate receptor (Oxgr1), showed both a functional and a binding response to LTE4 in these transfectants. We generated Gpr99−/− and Gpr99/Cysltr1/Cysltr2−/− mice for comparison with WT and Cysltr1/Cysltr2−/− mice. Strikingly, GPR99 deficiency in the Cysltr1/Cysltr2−/− mice virtually eliminated the vascular leak in response to the cys-LT ligands, indicating GPR99 as a potential CysLT1R active in the Cysltr1/Cysltr2−/− mice. Importantly, the Gpr99−/− mice showed a dose-dependent loss of LTE4-mediated vascular permeability, but not to LTC4 or LTD4, revealing a preference of GPR99 for LTE4 even when CysLT1R is present. As LTE4 is the predominant cys-LT species in inflamed tissues, GPR99 may provide a new therapeutic target.

Leukotriene C4 (LTC4), LTD4, and LTE4, collectively called cysteinyl leukotrienes (cys-LTs), are proinflammatory mediators derived from arachidonic acid through the 5-lipoxygenase/LTC4 synthase pathway (1, 2). Intracellularly synthesized LTC4 is exported via multidrug resistance-associated proteins and is rapidly metabolized in the extracellular space by cleavage removal of glutamic acid and then glycine to LTD4 and LTE4, respectively. The type 1 cys-LT receptor (CysLT1R) is the high affinity receptor for LTD4, whereas the type 2 receptor (CysLT2R) can bind both LTC4 and LTD4 with one-log less affinity than that of CysLT1R for LTD4 in transected cells (3, 4). The cys-LTs are mediators of bronchial asthma on the basis of their potent bronchoconstrictive activity via the CysLT1R in pharmacologic studies and the clinical effectiveness of CysLT1R antagonists (5, 6).

LTE4, the most abundant cys-LT at sites of inflammation due to its stability (7, 8), was only a weak agonist for the CysLT1R and CysLT2R in studies with transfectants (3, 4), suggesting that it might lack agonist activity. In contrast, pretreatment with LTE4, but not LTC4 or LTD4, enhanced the response of guinea pig trachea (9) or human bronchial smooth muscle to histamine (10). Furthermore, in limited studies, LTE4 was comparable with LTC4 or LTD4 in eliciting a vascular leak in skin guinea pigs (11) or humans (12), and inhalation of LTE4, but not LTD4, by patients with asthma elicited an accumulation of eosinophils and basophils into the bronchial mucosa (13, 14). These observations strongly supported an agonist function for LTE4 likely mediated by a receptor separate from those that are activated by its short-lived precursors (15).

To seek an additional receptor for the cys-LTs, we generated mice lacking the now classical CysLT1R, R and CysLT2R described by Evans and colleagues (3, 4) for humans and then by others for mice (16–18). As compared with WT mice, the Cysltr1/Cysltr2−/− strain on a BALB/c background showed similar dose-dependent edema responses to intradermal injection of LTC4 or LTD4 into the ear, indicating the presence of another receptor. Unexpectedly, LTE4 elicited more edema in the Cyslt1/Cysltr2−/− mice than LTD4 or LTC4 at the same dose and showed a 64-fold increase in potency in the Cysltr1/Cysltr2−/− mice as compared with WT mice, revealing a ligand preference for an unidentified receptor (19). Such a receptor could be important in asthma both because there is heterogeneity to the benefit of CysLT1R antagonists (20, 21) and because LTE4, unlike transient LTC4 and LTD4, is sustained at levels that even...
provide a urinary biomarker for the 5-lipoxygenase/LTC4 synthase pathway (22).

We have now identified GPR99, previously recognized as a G protein-coupled receptor for oxoglutarate (Oxgr1) (23), as CysLT1R, the LTE4-selective receptor functioning in the Cysltr1/Cysltr2\(^{-/-}\) strain. We identified GPR99 using a reporter gene expression assay to screen candidate cDNAs selected from the P2Y gene family that recognize dicarboxylic acids. We confirmed the nmol range binding of \(^{[3]H}\)LTE4 onto microsome membranes from GPR99-transfected cells. We then generated triple-deficient Gpr99/Cysltr1/Cysltr2\(^{-/-}\) mice to show the loss of permeability-enhancing function to all three cysteine leukotriene ligands in contrast to their sustained function in the presence of GPR99 but absence of the classical receptors in the Cysltr1/Cysltr2 \(^{-/-}\) strain. Finally, a dose-dependent loss of edema responses to LTE4, but not to LTC4 or LTD4, in Gpr99\(^{-/-}\) mice as compared with WT mice shows a preference of GPR99 for LTE4 even in the presence of the classic receptors.

**EXPERIMENTAL PROCEDURES**

**Reporter Gene Assay**—A trans-activator reporter gene assay system (Stratagene) was used to examine whether GPR91 or GPR99 can mediate LTE4-specific signaling in transfectants. pFACMV-trans-activator plasmids contain a fusion cDNA of the DNA-binding domain of GAL4 with an activation domain derived from CREB or Elk-1. When co-transfected with a gene that activates adenylate cyclase or MAPK, the activation domain from CREB or Elk-1 is phosphorylated, respectively, and binds to the GAL4 DNA-binding elements. A co-transfected pFA-Luc reporter plasmid that contains GAL4-binding reporter gene expression assay to screen candidate cDNAs and binds to the GAL4 DNA-binding elements. A co-transfected pFA-Luc reporter plasmid that contains GAL4-binding sites upstream of the firefly luciferase gene is then induced.

HeLa cells were transiently transfected with pCXN vector (a mammalian expression vector) control, pCXN-GPR91, or pCXN-GPR99 along with pFACMV-CREB or pFACMV-Elk-1 and pFACMV-Luc. After a 4-h incubation, the culture medium was replaced with serum-free DMEM. The next day, various concentrations of 1 \(\mu M\) succinate (100 \(\mu M\)), or oxoglutarate (100 \(\mu M\)) were added to the medium, and the cells were further incubated for 16 h. Then, the cells were lysed, and the firefly luciferase activity was measured. A Renilla luciferase plasmid was used as an internal control to assess the transfection efficiency as described in the manufacturer’s instructions (Promega).

**\(^{[3]H}\)LTE4 Binding Assay**—\(^{[3]H}\)LTE4 binding to the microsomal membranes from the CHO cell transfectants was assayed as described with modifications (16). \(^{[3]H}\)LTE4 was prepared from \(^{[3]H}\)LTD4 (PerkinElmer Life Sciences) by incubation with 10% FBS that contains dipeptidases, and the conversion was confirmed by reverse phase HPLC (24). 100 \(\mu g\) of membrane protein was incubated for 1 h at room temperature with varied concentrations of \(^{[3]H}\)LTE4. The LTE4-specific binding was determined by subtracting nonspecific binding, which was determined in the presence of 1 \(\mu M\) cold LTE4 from total binding. For competition assays, various concentrations of LTC4, LTD4, LTE4, MK-571, or oxoglutarate were added before incubation of the membrane protein with 2 nM \(^{[3]H}\)LTE4. When LTC4 and LTD4 were used as competitors, 50 \(\mu M\) serine borate and 20 \(\mu M\) l- penicillamine were included, respectively, to prevent conversion of the peptide adduct.

**Generation of Gpr99/Cysltr1/Cysltr2\(^{-/-}\)** Mice—Cysltr1/Cysltr2\(^{-/-}\) mice were generated by crossing breeding of BALB/c Cysltr1\(^{-/-}\) and Cysltr2\(^{-/-}\) mice as described (19). C57BL/6 Gpr99\(^{-/-}\) mice (the Knock-Out Mouse Project, University of California, Davis) were backcrossed on to the BALB/c background for four generations (N4). Because the Cysltr1 (on chromosome X) and Gpr99 (on chromosome 14) genes are on different chromosomes, BALB/c Gpr99/Cysltr1/Cysltr2\(^{-/-}\) mice were generated by crossing breeding as described for Cysltr1/Cysltr2\(^{-/-}\) mice. However, the Gpr99 and Cysltr2 genes are both on chromosome 14, and they are ~47 megabase pairs apart; we first bred BALB/c Gpr99\(^{-/-}\) mice with Cysltr2\(^{-/-}\) mice to obtain Gpr99\(^{-/-}\)/Cysltr2\(^{-/-}\) mice. We then bred Gpr99\(^{-/-}\)/Cysltr2\(^{-/-}\) mice with BALB/c WT mice to obtain Gpr99\(^{-/-}\)/Cysltr2\(^{-/-}\) (N5) mice with both mutant alleles on the same sister chromatid. These Gpr99\(^{-/-}\)/Cysltr2\(^{-/-}\) mice were intercrossed to obtain Gpr99/Cysltr2\(^{-/-}\) mice. Finally, Gpr99/Cysltr1\(^{-/-}\) mice were crossed with Gpr99/Cysltr2\(^{-/-}\) mice to obtain the triple null (Gpr99/Cysltr1/Cysltr2\(^{-/-}\)) strain. Gpr99\(^{-/-}\) and Gpr99/Cysltr1/Cysltr2\(^{-/-}\) mice were viable and had no apparent abnormalities based on fertility, behavior, and gross findings at autopsy. 2–4-month-old, male Gpr99\(^{-/-}\)/Cysltr1/Cysltr2\(^{-/-}\), and Gpr99/Cysltr1/Cysltr2\(^{-/-}\) mice were used. WT littersmates from the intercrossing of Gpr99\(^{-/-}\) or Gpr99/Cysltr1/Cysltr2\(^{-/-}\) mice were used as controls.

**Pharmacologic Assessment of Cys-LT-induced Ear Edema**—Mice received intradermal injections of various concentrations of LTD4, LTD4, or LTE4 in 25 \(\mu l\) of saline/dimethyl sulfoxide (DMSO) (vehicle) in the right ear and 25 \(\mu l\) of vehicle in the left ear. At 0, 30, 60, 120, and 240 min after the intradermal injection, ear thickness was measured with calipers (Dyer Co.). The results are presented as the difference of ear thickness between the right ear and left ear. All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

**Statistics**—Results were expressed as mean ± S.E. Student’s t test was used for the statistical analysis. A value of \(p < 0.05\) was considered significant.

**RESULTS AND DISCUSSION**

**Identification of a Candidate cDNA Encoding a Receptor for LTE4 Activation**—Based on their amino acid sequence, CysLT1R and CysLT2R belong to the nucleotide P2Y G protein-coupled receptor family (25). Within the P2Y4 subgroup, GPR91 and GPR99 do not bind to nucleotides but do bind to the glucose metabolism intermediates, succinate and oxoglutarate (\(\alpha\)-ketoglutarate), respectively (23). Because both succinate and oxoglutarate are dicarboxylic acids, and two carboxylic acid groups are also present in LTE4, we used a trans-activator reporter gene assay system to examine whether GPR91 or GPR99 can mediate LTE4-specific signaling in transfectants. Cells transfected with pCXN-GPR91 or a control vector alone did not respond to LTE4 in either assay. In contrast, cells transfected with pCXN-GPR99 responded to LTE4 in a dose-dependent manner in both assays beginning at a submicromolar concentration (Fig. 1A). As expected, oxoglutarate...
We then sought to show that GPR99 directly bound [3H]LTE4 using microsomal membranes from CHO cells transiently expressing GPR99. The [3H]LTE4 binding in the absence and presence of cold LTE4 revealed a specific dose-dependent binding to GPR99 with a Kd of 2499 PM and a Bmax of 274.6 fmol/mg of protein (Fig. 1B). Essentially no specific binding was obtained with vector-only transfected cells. By competition assays with 2 nM [3H]LTE4, cold LTE4 provided a dose-dependent inhibition to GPR99 transfectants (Fig. 1C). LTE4 showed 80% inhibition at 10 nM, whereas inhibition at this concentration was 60% for LTC4 and for oxoglutarate, and negligible for MK-571, a CysLT1R antagonist. Oxoglutarate completely inhibited the LTE4-elicited luciferase reporter gene and [3H]LTE4 binding assays. A, LTE4-elicited, CREB- or Elk-1-mediated luciferase expression in HeLa cells transfected with GPR99 (black columns) and vector (white columns). OG, oxoglutarate. B, specific [3H]LTE4 binding to 100 μg of microsomal membrane proteins from GPR99 (closed squares) and vector control (open squares) transfectants. Inset, Scatchard plot analysis for GPR99 transfectants is shown. C, competition for [3H]LTE4 binding (2 nM) by LTE4 (squares), oxoglutarate (circles), LTC4 (triangles), LTD4 (inverted triangles), and MK-571 (diamonds). Data are representative of two independent experiments.

GPR99 Mediates LTE4-Induced Ear Edema in the Cysltr1/Cysltr2−/− and in WT Mice—The Cysltr1/Cysltr2−/− strain exhibits a markedly augmented swelling response to intracutaneous administration of LTE4 and an essentially intact response to LTC4 and LTD4 (19). Thus, we sought to verify that GPR99 is a bona fide third receptor with a preference for LTE4 by deleting it from this strain and studying skin swelling responses in the resultant triple-null mice. LTE4 (0.5 nmol/site) was injected intradermally into the ear of BALB/c WT, Gpr99−/−, Cysltr1/Cysltr2−/−, and Gpr99/Cysltr1/Cysltr2−/− mice, and tissue edema was assessed by measuring ear thickness at 0, 30, 60, 120, 180, and 240 min after the injection. Net ear swelling after correction for the left vehicle-injected ear peaked in WT mice at 30 min, and in Cysltr1/Cysltr2−/− mice, there was an enhanced peak response at 30 min (Fig. 2A) as reported previously (19). Gpr99−/− mice showed essentially the same response as WT mice, indicating an absence of a GPR99 role at this ligand dose. In contrast, Gpr99/Cysltr1/Cysltr2−/− mice had a more than 90% loss of the peak response at 30 min as compared with WT, Gpr99−/−, and Cysltr1/Cysltr2−/− mice (p < 0.01), indicating that the enhanced LTE4-induced ear edema in Cysltr1/Cysltr2−/− mice is mediated through GPR99.

To assess the dose dependence of the GPR99 response in the absence and presence of the classical receptors, we reduced the dose of LTE4 by 64-fold (0.008 nmol/site). The peak edema at 30 min with this minimal dose in WT mice was reduced by about one-half and again was exceeded by that in the Cysltr1/Cysltr2−/− mice (Fig. 2B). As expected, the Gpr99/Cysltr1/Cysltr2−/− mice showed essentially no edema response at any time point due to the absence of all three receptors. Of note, at this dose, Gpr99−/− mice had a markedly reduced peak response (p < 0.05), suggesting that GPR99 is the major LTE4 receptor when the ligand dose is below the threshold needed to activate CysLT1R or CysLT2R in vivo. To obtain further evidence supporting this suggestion, an intermediate dose of 0.0625 nmol of LTE4 was used to elicit the vascular leak. The enhanced edema relative to WT mice in the Cysltr1/Cysltr2−/− strain was again virtually abolished in the Gpr99/Cysltr1/Cysltr2−/− strain (Fig.
The peak response in the Gpr99−/− strain at 30 min was also diminished to almost one-half that of the WT and absent beyond this time point. Taken together, the dose-response study with LTE₄ establishes GPR99 as the preferred LTE₄ receptor at one-eighth and one-sixty-fourth of the 0.5-nmol dose in the presence of the classical CysLTRs and at all doses when the classical receptors are absent.

**GPR99 Mediates LTC₄ and LTD₄-induced Ear Edema in the Cysltr1/Cysltr2−/− Mice**—To assess whether GPR99 mediates the permeability-enhancing action of the other cys-LT ligands, we injected LTC₄ and LTD₄ at 0.5 or 0.008 nmol/site intradermally into the ear of BALB/c WT, Gpr99−/−, Cysltr1/Cysltr2−/−, and Gpr99/Cysltr1/Cysltr2−/− mice. With the high dose of LTC₄, WT mice had an ear edema response with peak at 30–60 min, and Gpr99−/− mice had a similar response (Fig. 3A). Although Cysltr1/Cysltr2−/− mice had a slightly reduced peak response to LTC₄ at 30–60 min as compared with WT mice, the Gpr99/Cysltr1/Cysltr2−/− mice had a markedly reduced peak at 30 min, showing GPR99 to be a third cys-LT receptor. With the low dose of LTC₄, WT mice had a reduced ear edema response with peak at 30–60 min, and Gpr99−/− mice had a similar response (Fig. 3B). As expected, although Cysltr1/Cysltr2−/− and WT mice had a similar peak response at 30 min, the Gpr99/Cysltr1/Cysltr2−/− mice had no response to the low dose LTC₄ at any time point. These results indicate that GPR99 can mediate LTC₄-induced edema responses in the absence of CysLT₁R and CysLT₂R.

With the 0.5 nmol/site of LTD₄, WT mice and Gpr99−/− mice showed peak edema responses at 30–60 min (Fig. 3C). The Cysltr1/Cysltr2−/− mice showed a reduced peak response at 30 min, whereas there was virtually no response to LTD₄ in the Gpr99/Cysltr1/Cysltr2−/− mice. With the low dose of LTD₄, WT mice and Gpr99−/− mice showed reduced but similar peak edema responses at 30–60 min (Fig. 3D). Although Cysltr1/Cysltr2−/− and WT mice had a similar peak response at 30–60 min, the Gpr99/Cysltr1/Cysltr2−/− mice had virtually no response. Thus, GPR99 can mediate LTD₄-induced edema responses in the absence of CysLT₁R and CysLT₂R.

The findings in the triple receptor-deficient mice indicate GPR99 as a potential CysLT₃R, a receptor capable of responding to each of the cys-LTs in the absence of the classical receptors, CysLT₁R and CysLT₂R. A preference or greater sensitivity of GPR99 for LTE₄ is shown by the enhanced response of the Cysltr1/Cysltr2−/− mice as compared with WT mice but not to LTC₄ or LTD₄. This is supported by the reduction of the permeability response to low and intermediate doses of LTE₄ in the Gpr99−/− strain. At a high dose of LTE₄, the GPR99-deficient strain is not impaired due to the dominance of the CysLT₂R over GPR99. The strength and sensitivity of the LTE₄-mediated vascular leak in the absence of the classical receptors implies their negative regulation for GPR99 function or sensitivity.

The human GPR99 gene was originally identified as an orphan G protein-coupled receptor (GPCR) with homology to a P2Y nucleotide receptor subfamily by searching the human genomic databases with known nucleotide receptors (26). Human GPR99 shares 36% identical amino acids both with the ATP receptor, P2Y₁, and with GPR91, which had been identified as another orphan GPCR at that time. Mouse GPR99 shares 85% identical amino acids with human GPR99. The motif YXXVTRPL, which is unique to the P2Y₁ subfamily, and the motif HXX(R/K), which is found in all nucleotide-binding GPCRs, are conserved between mouse and human GPR99. Mouse GPR99 is 33% identical to mouse GPR91, 32% identical to mouse CysLT₃R, and 27% identical to mouse CysLT₁R. The HXX(R/K) motif is conserved in GPR99, GPR91, CysLT₁R, and CysLT₂R, and the YXXVTRPL motif is conserved only in GPR99 and GPR91 (supplemental Fig. 1). The positively charged arginine residues in both motifs are required for succinate-elicited GPR91 activation as assessed by site-directed mutagenesis and functional reporter gene assays (23). Another arginine (position 95 in mouse GPR91 and position 99 in human GPR91) and a histidine (position 99 in mouse GPR91 and position 103 in human GPR91) are also required for succinate-elicited GPR91
activation (supplemental Fig. 1). Because these four amino acids are conserved in GPR99, they may be important in binding dicarboxylic acid ligands. Particularly, the arginine in the YXVTRPL motif, which is lacking in CysLT1R and CysLT2R, might account for the preference of GPR99 for LTE4.

Northern blot analysis with human tissues revealed that GPR99 mRNA is expressed in kidney and placenta (26). By a quantitative RT-PCR, human GPR99 mRNA is predominantly expressed in trachea, salivary glands, kidney, fetal brain, and lung and highly expressed in umbilical cord blood-derived mast cells (27). A quantitative RT-PCR analysis in the mouse revealed that GPR99 mRNA is highly expressed in kidney, testis, and smooth muscle (23). Given that CysLT1R and CysLT2R are also expressed on smooth muscle cells in airways and blood vessels, expression and function of GPR99 on smooth muscle may be affected by the presence of CysLT1R and CysLT2R.

LTE4 has a sufficient biologic half-life to be excreted into the urine (30), and urinary LTE4 excretion is significantly increased in spontaneous acute asthma flares (22). Asthmatic individuals show LTE4-induced bronchoconstriction as an aerosol in the same concentration range as LTD4 and LTC4 (31, 32), and LTE4 potentiates airway hyperresponsiveness to histamine (33). Furthermore, patients with aspirin-exacerbated respiratory disease (AERD) have both persistently high basal urinary levels of LTE4 and a marked (~10-fold) further increment in urinary LTE4 excretion with aspirin challenge (34). Treatment with montelukast, a CysLT1R antagonist, improves lung function and quality of life of patients with AERD (6), but its beneficial effect is limited and heterogeneous among patients (35). GPR99 may be involved in the chronicity of signs and symptoms, such as nasal polyps, in patients with AERD.

FIGURE 3. GPR99 mediates LTC4− and LTD4− induced ear edema in Cysltr1/Cysltr2−/− mice. WT (squares), Gpr99−/− (diamonds), Cysltr1/Cysltr2−/− (closed circles), and Gpr99/Cysltr1/Cysltr2−/− (open circles) mice received intradermal injections of 0.5 (A) and 0.008 nmol (B) of LTC4 or 0.5 (C) and 0.008 nmol (D) of LTD4 in the right ear and vehicle in the left ear. Ear thickness was measured at the indicated times after the injection. Results are expressed as the mean ± S.E. (3 mice per group) of the net differences in the thickness.

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