Functional recognition of a distinct receptor preferential for leukotriene E₄ in mice lacking the cysteinyl leukotriene 1 and 2 receptors

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Contributed by K. Frank Austen, September 10, 2008 (sent for review August 25, 2008)

The cysteinyl leukotrienes (cys-LTs) are a family of potent lipid mediators of inflammation derived from arachidonic acid. Activation of certain cell types results in the biosynthesis and export of leukotriene (LT) C₄, which then undergoes extracellular metabolism to LTD₄ and LTE₄. LTE₄, the most stable cys-LT, is only a weak agonist for the defined type 1 and type 2 cys-LT receptors (CysLT₁R and CysLT₂R, respectively). We had recognized a greater potency for LTE₄ than LTC₄ or LTD₄ in constricting guinea pig trachea *in vitro* and comparable activity in eliciting a cutaneous wheal and flare response in humans. Thus, we hypothesized that a vascular permeability response to LTE₄ in mice lacking both the CysLT₁R and CysLT₂R could establish the existence of a separate LTE₄ receptor. We now report that the intradermal injection of LTE₄ into the ear of mice deficient in both CysLT₁R and CysLT₂R elicits a vascular leak that exceeds the response to intradermal injection of LTC₄ or LTD₄, and that this response is inhibited by pretreatment of the mice with pertussis toxin or a Rho kinase inhibitor. LTE₄ is ≈64-fold more potent in the CysLT₁R/CysLT₂R double-deficient mice than in sufficient mice. The administration of a CysLT₁R antagonist augmented the permeability response of the CysLT₁R/CysLT₂R double-deficient mice to LTC₄, LTD₄, and LTE₄. Our findings establish the existence of a third receptor, CysLT_ER, that responds preferentially to LTE₄, the most abundant cys-LT in biologic fluids, and thus reveal a new target for therapeutic intervention.

inflammation | lipid mediator | knockout mice

To recognize a role for the cysteinyl leukotrienes (cys-LTs) and their receptors in inflammation, we initially developed mice lacking the critical biosynthetic enzyme, leukotriene C₄ synthase (LTC₄S), which forms leukotriene (LT) C₄ by conjugation of reduced glutathione to LTA₄. LTA₄ is generated from arachidonic acid released from phospholipids of the outer nuclear membrane by cytosolic phospholipase A₂α during cell activation. In the presence of 5-lipoxygenase (5-LO) and the 5-LO-activating protein (FLAP), the arachidonic acid is converted sequentially to 5-hydroperoxyeicosatetraenoic acid and LTA₄ (1, 2). Both LTC₄S and FLAP are integral proteins of the outer nuclear membrane and function as trimers (3–5) in the tightly regulated intracellular synthesis of LTC₄. After its export via an energy-dependent step that requires multidrug resistance-associated proteins 1 and 4, LTC₄ is metabolized by cleavage removal of glutamic acid and then glycine to provide LTD₄ and LTE₄, respectively. In a passive cutaneous anaphylaxis model, mice lacking LTC₄S (*Ltc4s*^{−/−}) exhibited a significant reduction in vascular leak after local sensitization of ear mast cells with specific IgE and systemic challenge with antigen (6). These findings revealed a permeability-enhancing function for cys-LTs comparable to that of the amines stored in the mast cell secretory granules. These *Ltc4s*^{−/−} mice also were significantly protected against bleomycin-induced pulmonary fibrosis and antigen-induced allergic inflammation of the lung (7, 8).

To further analyze the cys-LT-mediated inflammatory pathways, we generated mice deficient in the type 1 cys-LT receptor

(CysLT₁R) and the type 2 cys-LT receptor (CysLT₂R) based on the prior cloning of these seven-transmembrane, G protein-coupled human receptors by others (9, 10). CysLT₁R and CysLT₂R are 38% homologous in their amino acid sequences. The rank order of affinities of the cys-LTs for the CysLT₁R and CysLT₂R based on transfected cells is LTD₄ > LTC₄ > LTE₄ and LTC₄ = LTD₄ > LTE₄, respectively (9–12). CysLT₁R-deficient (*Cysltr1*^{−/−}) and CysLT₂R-deficient (*Cysltr2*^{−/−}) mice each showed a significant reduction in vascular permeability in the passive cutaneous anaphylaxis model comparable to that in the *Ltc4s*^{−/−} mice (13, 14), suggesting either sequential functions in the microvasculature or heterodimerization of these receptors. Bleomycin-induced fibrosis in *Cysltr2*^{−/−} mice, but not in *Cysltr1*^{−/−} mice, was as reduced as that in *Ltc4s*^{−/−} mice, revealing a requirement of CysLT₂R for this complex injury. In contrast, neither the *Cysltr1*^{−/−} nor the *Cysltr2*^{−/−} mice were protected from antigen-induced pulmonary inflammation, suggesting that additional receptor(s) might account for the protection observed in the *Ltc4s*^{−/−} mice (unpublished data).

We had observed previously that LTE₄ was more potent than LTC₄ or LTD₄ in contracting the guinea pig trachea *in vitro* (15, 16) and was comparable to LTC₄ or LTD₄ in eliciting a dermal wheal and flare response in humans (17). Others noted that inhalation of LTE₄ but not LTD₄ by patients with asthma elicited an influx of eosinophils and basophils into the bronchial mucosa (18, 19). Furthermore, patients with aspirin-exacerbated respiratory disease, characterized by bronchial asthma, nasal polyps, and marked overproduction of the cys-LTs as defined by urinary excretion of *N*-acetyl-LTE₄, responded with enhanced bronchoconstriction to inhalation of LTE₄ relative to their responses to LTC₄ or histamine, as compared with the potency of these agonists in asthmatic patients without aspirin-exacerbated respiratory disease (20). These preferential agonist functions of LTE₄ were not explained by the pharmacologic properties of the CysLT₁R and CysLT₂R.

To seek an additional receptor(s) for the cys-LTs, we generated CysLT₁R/CysLT₂R double-deficient (*Cysltr1/Cysltr2*^{−/−}) mice and examined the agonist function of the cys-LTs by intradermal injection into the ear. We found that the *Cysltr1/Cysltr2*^{−/−} mice expressed a previously unrecognized receptor that mediated a greater vascular leak in response to LTE₄ than to LTD₄ or LTC₄. The increased sensitivity of the *Cysltr1/Cysltr2*^{−/−} mice compared with WT mice was ≈64-fold by

Author contributions: A.M., Y.K., and K.F.A. designed research; A.M., Y.K., and W.X. performed research; A.M., Y.K., W.X., and K.F.A. analyzed data; and A.M., Y.K., and K.F.A. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0808993105/DCSupplemental.

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Fig. 1. Effects of CysLTR deficiency on LTC₄, LTD₄, and LTE₄-induced ear edema. WT (closed squares, $n = 7$), *Cysltr1/Cysltr2*^{-/-} (open circles, $n = 5$), *Cysltr1*^{-/-} (diamonds, $n = 5$), and *Cysltr2*^{-/-} (triangles, $n = 3$) mice received intradermal injections of 0.5 nmol (in 25 μ l vehicle) of LTC₄ (A), LTD₄ (B), or LTE₄ (C) in the right ear and 25 μ l vehicle in the left ear. Ear thickness was measured with calipers at the indicated times after the injection. Results are expressed as the mean $\pm$ SE of the differences between the thickness of the right and left ears at each time point and are combined from two independent experiments. Error bars are too small to be visible compared with the physical size of the symbol. *, $P < 0.01$ vs. WT mice is presented in the plot for 30, 60 and 240 min.

dose-dependent analysis, thereby revealing a profound negative regulation of the novel receptor for LTE₄ by the two known receptors with a preference for LTD₄ and LTC₄. We then found that pretreatment of *Cysltr1/Cysltr2*^{-/-} mice with a selective CysLT₁R antagonist, MK-571, further increased the permeability response of these mice to each of the three cys-LTs. Thus, these studies uncover a distinct receptor with a preference for LTE₄, which we term CysLT_ER, that could be relevant to pathobiology mediated by the most stable cys-LT, LTE₄ (21, 22).

Results and Discussion

Enhanced LTE₄-Elicited Ear Edema in *Cysltr1/Cysltr2*^{-/-} Mice Indicates the Presence of a Novel Receptor. To examine whether any cys-LTs mediate an increase in vascular permeability in the absence of their known receptors, LTC₄, LTD₄, and LTE₄ (0.5 nmol per site) were injected intradermally into the ear of WT, *Cysltr1*^{-/-}, *Cysltr2*^{-/-}, and *Cysltr1/Cysltr2*^{-/-} mice, and tissue edema was assessed by measuring ear thickness at 0, 30, 60, 120, 180, 240, and 300 min after the injection. Ear edema induced by LTC₄ and that induced by LTD₄ in WT mice were similar, peaking at 30 min and returning to baseline at 300 min (Fig. 1A and B). LTC₄- and LTD₄-induced ear edema in *Cysltr1*^{-/-} mice both were reduced by 50–60% at the peak compared with levels in WT mice and returned to baseline 60 min earlier. LTC₄- and LTD₄-induced ear edema in *Cysltr2*^{-/-} mice showed a delayed peak response at 60 min that was comparable to the earlier peak response in WT mice (Fig. 1A and B). These results in mice lacking a single receptor indicate that about 50% of LTC₄- and LTD₄-induced ear edema is mediated by CysLT₁R and suggest that CysLT₂R may be a negative regulator of the CysLT₁R-mediated response. Of particular note, *Cysltr1/Cysltr2*^{-/-} mice responded to intradermal LTC₄ and LTD₄, respectively, with a vascular leak similar to that of WT mice (Fig. 1A and B), revealing an additional receptor that is distinct from CysLT₁R and CysLT₂R.

The ear edema in response to intradermal injection of LTE₄ in WT mice with a peak at 30 min and resolution at 300 min was similar to the edema induced by LTC₄ and LTD₄ at the same 0.5-nmol dose (Fig. 1C). However, unlike the reduced response to LTC₄ or LTD₄, LTE₄-induced ear edema in *Cysltr1*^{-/-} mice was comparable in magnitude to that in WT mice, indicating a CysLT₁R-independent action. The LTE₄-induced ear edema in *Cysltr2*^{-/-} mice showed a peak response similar to that in WT mice, but the resolution was delayed; this finding suggested an inhibitory role directed to a CysLT₁R-independent LTE₄ receptor. Unexpectedly, *Cysltr1/Cysltr2*^{-/-} mice showed a significantly enhanced peak response ($11.1 \pm 0.1 \times 10^{-2}$ mm, $n = 5$) to LTE₄ injection at 30 min compared with WT mice ($8.5 \pm 0.32 \times 10^{-2}$ mm, $n = 7$), and the response remained evident even at 300 min

($5.5 \pm 0.32 \times 10^{-2}$ mm), when the response in WT mice had resolved (Fig. 1C).

To assess whether the 30% increase in permeability in response to LTE₄ in the *Cysltr1/Cysltr2*^{-/-} mice compared with WT mice was limited by being a near-maximum response at the fixed 0.5-nmol dose used for the three ligands, we examined the dose–response to LTE₄ (0.5, 0.125, 0.03, and 0.008 nmol per site) in WT and *Cysltr1/Cysltr2*^{-/-} mice. LTE₄ elicited ear edema in both WT and *Cysltr1/Cysltr2*^{-/-} mice in a dose-dependent manner, with a significantly greater response in the *Cysltr1/Cysltr2*^{-/-} mice at each dose (Fig. 2). The increased ear swelling at 30 min in the *Cysltr1/Cysltr2*^{-/-} mice at the dose of 0.008 nmol per site was essentially equal to that in WT mice at the dose of 0.5 nmol per site, indicating that *Cysltr1/Cysltr2*^{-/-} mice are ≈ 64 -fold more sensitive to LTE₄ for ear edema compared with WT mice. The persistence of edema to 300 min in the *Cysltr1/Cysltr2*^{-/-} mice with each dose, compared with full resolution in the WT mice, indicates that the augmented 30-min peak may be influenced by reduced clearance.

To assess the rank order of agonist action for this novel receptor, we examined the dose–response to intradermal injections of LTC₄ (0.5, 0.125, and 0.03 nmol per site) and LTD₄ (0.5, 0.125, and 0.03 nmol per site) in WT and *Cysltr1/Cysltr2*^{-/-} mice. Both WT and *Cysltr1/Cysltr2*^{-/-} mice showed dose-dependent responses to LTC₄ and LTD₄, and the peak responses at 30 min for each dose of the ligand in the *Cysltr1/Cysltr2*^{-/-} mice were essentially similar to the responses in WT mice, although prolonged [supporting information (SI) Fig. S1]. The finding that the peak responses to LTC₄ and LTD₄ persisted until 60 min in the *Cysltr1/Cysltr2*^{-/-} mice may reflect conversion to LTE₄, since we could detect LTC₄ and LTE₄ with a 1:3 molar ratio in extracts of ear tissues from two mice 60 min after injection of 400 pmol of LTC₄ by reverse-phase HPLC ($\approx 10\%$ of recovery), whereas no cys-LTs were detected in the saline-injected ears. These results strongly indicate that a separate receptor for LTC₄, LTD₄, and LTE₄ can be recognized in the absence of the two known receptors and that the rank order of agonist action for this receptor, assessed by increased vascular permeability, is LTE₄ > LTC₄ = LTD₄. This distinct receptor is termed CysLT_ER to indicate its functional ligand selectivity.

Histologic Assessment of LTE₄-Elicited Ear Edema. To determine whether the LTE₄-elicited ear thickening at the peak and during the resolution phase is due solely to plasma protein extravasation, ear tissues were harvested for histologic analysis 30 and 240 min after LTE₄ intradermal injection. LTE₄ caused expansion of the extracellular space in an injected side of the ear tissue but no detectable cellular infiltration in either WT or *Cysltr1/Cysltr2*^{-/-} mice (Fig. 3). The LTE₄-elicited ear thickening measured from

Fig. 4. Effect of MK-571 on LTC₄-, LTD₄-, and LTE₄-induced ear edema in WT and CysLTR-deficient mice. WT (closed squares, $n = 4$), *Cysltr1*^{-/-} (diamonds, $n = 4$), *Cysltr2*^{-/-} (triangles, $n = 4$), and *Cysltr1/Cysltr2*^{-/-} (open circles, $n = 4$) mice received intradermal injections of 0.5 nmol (A and B) or 0.008 nmol (C) of LTE₄ or 0.5 nmol of LTC₄ (D) or LTD₄ (E) in the right ear and vehicle in the left ear without (dotted lines) or with (solid lines) i.v. injection of MK-571 (10 mg/kg) 30 min previously. Results are expressed as the mean $\pm$ SE of the differences between the thickness of the right and left ears at each time point and are combined from two independent experiments. Some error bars are too small to be visible compared with the physical size of the symbol. Experiments for A and B were carried out at the same time. *, $P < 0.01$ vs. mice without MK-571 within each individual strain presented for 30, 60, and 240 min.

LTE₄-stimulated ERK activation in CHO cells and is responsible for a marked potentiating effect of intranasal LTE₄ on aerosol antigen-induced airway mucosal eosinophilia and goblet cell hyperplasia in sensitized mice. This effect of LTE₄ is inhibited by P2Y₁₂ receptor antagonism with clopidogrel (J. Boyce, personal communication). In contrast, the enhanced response to LTE₄ in the *Cysltr1/Cysltr2*^{-/-} mice was not affected by pretreatment with clopidogrel, indicating that the responsible receptor is not P2Y₁₂ (Fig. 5E).

Our finding of a functional CysLT_{ER} in the ear of *Cysltr1/Cysltr2*^{-/-} mice that mediates an increase in vascular permeability to each cys-LT with a preference for LTE₄ highlights the complexity of the cys-LT system *in vivo*. The permeability increase in WT mice at the dose of 0.5 nmol per site was comparable for the three cys-LTs, but the response to LTE₄ was not inhibited in the *Cysltr1*^{-/-} mice, implying recognition by CysLT_{ER} (Fig. 1). The vascular leak in the *Cysltr2*^{-/-} mice was prolonged but not increased in peak for each of the three cys-LTs, suggesting that CysLT_{2R} is a negative regulator of CysLT_{1R} and of CysLT_{ER}. The role of CysLT_{2R} as a negative regulator of CysLT_{1R} has been previously observed in proliferation assays of culture-derived human and mouse mast cells (29). The finding that the peak response to LTE₄ at 30 min was enhanced in *Cysltr1/Cysltr2*^{-/-} mice, but not in either *Cysltr1*^{-/-} or *Cysltr2*^{-/-} mice, indicates that these receptors may each be intrinsic negative regulators of CysLT_{ER} function.

The introduction of the prototype "lukast" inhibitor uncovered yet another level of complexity. Although MK-571 reduced the permeability effect of LTE₄ in WT mice, it did not inhibit the effect in *Cysltr1*^{-/-} mice, and it increased the permeability response to LTE₄ in *Cysltr2*^{-/-} mice to the level of *Cysltr1/Cysltr2*^{-/-} mice (Fig. 4 A and B). Importantly, the enhanced permeability response in *Cysltr1/Cysltr2*^{-/-} mice not only to LTE₄ but also to LTC₄ and LTD₄ in the presence of MK-571

indicates the presence of a class-related negative regulation independent of expression of CysLT_{1R} and CysLT_{2R} (Fig. 4 C–E). Such an array of intrinsic and induced negative regulation may have evolved because LTE₄, the most stable cys-LT, persists in inflammatory responses in human and mouse tissues in contrast to its labile precursors, LTC₄ and LTD₄ (21, 30, 31). In view of the comparable vascular potency of LTE₄ to that of LTC₄ and LTD₄ in mouse ear skin (Fig. 1) and human forearm skin (17), it is possible that constitutive expression of CysLT_{ER} may be harmful to the host homeostasis, and hence highly regulated. However, the fact that CysLT_{1R}, and probably CysLT_{2R} as well, is rapidly internalized in an agonist-dependent manner (32) could provide settings that favor the presence of CysLT_{ER} in acute and chronic inflammation.

Antagonists selective for CysLT_{1R} alone or for both CysLT_{1R} and CysLT_{2R} have been used to implicate a separate receptor refractory to these inhibitors for contractions of pulmonary artery from humans (33, 34) and pigs (35). In the latter study, both LTC₄ and LTD₄ contracted intact and rubbed rings from porcine pulmonary artery in a dose-dependent manner that was resistant to either a CysLT_{1R} or a dual-receptor antagonist. That LTE₄ had a negligible agonist effect in this assay even at the highest dose of 1 μ M indicates that functional CysLT_{ER} was not involved. Recently, Paruchuri *et al.* reported that LTE₄ can stimulate human mast cells to produce prostaglandin D₂ through the nuclear receptor peroxisome proliferator-activated receptor- γ with more potency than LTD₄, and that this response was intact after lentiviral knockdown of the CysLT_{1R} but blocked by MK-571 (36). Lentiviral knockdown of the P2Y₁₂ receptor blocked this LTE₄ response (J. Boyce, personal communication), thereby distinguishing this receptor from CysLT_{ER}.

Our finding in mice of the presence of a novel LT receptor with a preference for LTE₄ relative to LTC₄ or LTD₄ raises the question of whether such a putative receptor has been recog-

were stained by the chloroacetate esterase reaction to identify mast cells, and neutrophils and were counterstained with hematoxylin.

Measurement of cys-LTs from Ear Tissues After LTC₄ Injection. Mice received intradermal injections of 400 pmol LTC₄ in 20 μ l saline/DMSO in the right ear and 20 μ l saline/DMSO (vehicle) in the left ear. At 60 min after the intradermal injection, ear tissues were isolated and homogenated with a Tissue-Tearor homogenizer (Biospec Products) in 1 ml of 50 mM Hepes buffer, pH 7.9, containing 50% methanol and 200 ng prostaglandin B₂. The tissue homogenates were centrifuged at 1,000 $\times$ g for 5 min at 4°C, and the supernatants were collected for measurements of cys-LTs by reverse-phase HPLC as previously described (40). Briefly, samples were applied to a C18 Ultrasphere RP column (Beckman Instruments) equilibrated with a solvent of methanol/ acetonitrile/water/acetic acid (10:15:100:0.2, vol/vol), pH 6.0 (solvent A). After injection of the sample, the column was eluted at a flow rate of 1 ml/min with a programmed concave gradient to 55% of the equilibrated solvent A and 45% solvent B (100% methanol) over 2.5 min. After 5 min, solvent B was

increased linearly to 75% over 15 min and was maintained at this level for an additional 15 min. The UV absorbance at 280 nm and the UV spectra were recorded simultaneously. The retention times for prostaglandin B₂, LTC₄, LTD₄, and LTE₄ were 20.4, 21.5, 23.2, and 24.5 min, respectively. LTC₄ and LTE₄ were quantitated by calculating the ratio of each peak area to the peak area of the internal standard prostaglandin B₂.

Statistics. Student's *t* test was used for the statistical analysis in cases in which the variance was homogeneous, and Welch's test was used when the variance was heterogeneous. For simplicity, statistical analyses were performed at 30 and 60 min for the peak and 240 min for resolution. A value of *P* < 0.05 was considered significant.

ACKNOWLEDGMENTS. We thank Juying Lai for technical assistance, Dr. Bing Lam for HPLC analysis of cys-LTs, and Drs. Joshua Boyce and Jonathan Arm for critical reading of the manuscript. This work was supported by National Institutes of Health grants AI-31599, AI-52533, HL-36110, and HL-82695.

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