Elucidation of signaling and functional activities of an orphan GPCR, GPR81

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Abstract GPR81 is an orphan G protein-coupled receptor (GPCR) that has a high degree of homology to the nicotinic acid receptor GPR109A. GPR81 expression is highly enriched and specific in adipocytes. However, the function and signaling properties of GPR81 are unknown because of the lack of natural or synthetic ligands. Using chimeric G proteins that convert Gi-coupled receptors to Gq-mediated inositol phosphate (IP) accumulation, we show that GPR81 can constitutively increase IP accumulation in HEK293 cells and suggest that GPR81 couples to the Gi signaling pathway. We also constructed a chimeric receptor that expresses the extracellular domains of cysteinyl leukotriene 2 receptor (CysLT2R) and the intracellular domains of GPR81. We show that the CysLT2R ligand, leukotriene D_4 (LTD4), is able to activate this chimeric receptor through activation of the Gi pathway. In addition, LTD4 is able to inhibit lipolysis in adipocytes expressing this chimeric receptor. These results suggest that GPR81 couples to the Gi signaling pathway and that activation of the receptor may regulate adipocyte function and metabolism. Hence, targeting GPR81 may lead to the development of a novel and effective therapy for dyslipidemia and a better side effect profile than nicotinic acid.—Ge, H., J. Weiszmann, J. D. Reagan, J. Gupte, H. Baribault, T. Gyuris, J-L. Chen, H. Tian, and Y. Li. **Elucidation of signaling and functional activities of an orphan GPCR, GPR81.** ***J. Lipid Res.*** **2008.** 49: **797–803.**

Supplementary key words G protein-coupled receptor • dyslipidemia • niacin • GPR109A • free fatty acid • HDL • adipocyte • diabetes

Nicotinic acid has been used for the treatment of dyslipidemia for >50 years. The drug improves multiple cardiovascular risk factors, including increase of HDL and reduction of VLDL, LDL, lipoprotein [a], and triglycerides (TGs), that overall result in a reduction in mortality (1). The receptor for nicotinic acid, termed GPR109A (HM74A in humans and PUMA-G in mice), identified in recent years couples to G proteins of the Gi family and is expressed mainly in adipocytes and immune cells (2–4). Although the precise mechanism of action for nicotinic acid is unknown, GPR109A-mediated inhibition of lipolysis in adipocytes, which results in a reduction in plasma FFA levels, is postulated to play an important role in the improvement of plasma lipid parameters (5, 6). Changes in plasma FFA levels may also lead to changes in the peripheral tissue responsiveness to insulin as well. There is an observed correlation between increased plasma FFA levels and type 2 diabetes as well as an increased risk of type 2 diabetes in individuals with high plasma FFA levels. Furthermore, increased plasma FFA levels can lead directly to the inhibition of insulin-dependent glucose disposal, increased hepatic gluconeogenesis, and impaired pancreatic islet functions (7). Indeed, it has been observed that short-term treatment with nicotinic acid and its analogs improves insulin action (6), while sustained reduction in plasma FFAs by treatment with the nicotinic acid analog acipimox improves the clinical course of type 2 diabetes (8). Therefore, targeting fat tissue and regulating adipocyte lipolysis may be a general strategy to regulate lipid and glucose homeostasis.

Despite its great efficacy in improving plasma lipid parameters, nicotinic acid treatment results in a number of undesirable side effects, including flushing in the face and upper body and gastrointestinal upset. Recently, it was reported that in rodents the activation of GPR109A in the epidermal Langerhans cells via the release of prostaglandins mediates the flushing side effect of nicotinic acid (9, 10). A number of different approaches have been taken to alleviate the side effects, including the use of an extended-release formulation (1), receptor-selective agonists/modulators that only activate the antilipolytic pathway while avoiding the activation of the immune-related flush-inducing pathway (11), and combination treatment of nicotinic acid with prostaglandin D_2 receptor 1 antagonists (12). Another alternative strategy is to investigate other adipocyte Gi-coupled G protein-coupled

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Abbreviations: CysLT2R, cysteinyl leukotriene 2 receptor; GPCR, G protein-coupled receptor; IP, inositol phosphate; LTD4, leukotriene D_4; PTX, pertussis toxin.

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receptors (GPCRs) that are either not expressed in immune cells or whose activation does not induce flushing.

GPR81 is an orphan GPCR that belongs to the same subfamily of receptors as GPR109A and GPR109B (13). GPR81 shares ~51% sequence identity with GPR109A and is colocalized in humans on the same chromosome, 12q24.31, as GPR109A and GPR109B (6). GPR81 expression was found to be highest in adipose tissue among a number of human tissues tested (2). Because no ligands have been described for GPR81, the signaling pathway for receptor activation as well as the functions of the receptor are largely unknown.

Here, using chimeric G proteins as well as chimeric cysteinyl leukotriene 2 receptor (CysLT2R) and GPR81 receptors, we demonstrate that GPR81 couples to Gi pathways for receptor activation. Furthermore, we show that activation of our chimeric receptor by leukotriene D_4 (LTD4) results in the inhibition of lipolysis in adipocytes. These results suggest that activation of adipocyte-specific GPR81 has the potential to promote a similar positive clinical outcome as nicotinic acid treatment, but without the skin-mediated flushing side effect.

MATERIALS AND METHODS

Tissue expression analysis of GPR81

GPR81 expression in tissues and differentiated adipocytes was analyzed using quantitative PCR. Mouse RNA was from Ambion FirstChoice Total RNA: Mouse Normal Tissue Survey Panel (catalog No. AM7800). Mouse 3T3-L1 preadipocytes were cultured and differentiated into adipocytes as described previously (14). The primers used for GPR81 were 5′-TCTTCCTGCCCCTGACAATC-3′, 5′-CCGTCTCAGGCTCCAAACA-3′, and 5′-6FAM-TCTTGTTCTGCTCGGTCAACG-BHQ1-3′; the primers for CysLT2R/GPR81 were 5′-CAACCATCCATCTCCGTATCAG-3′, 5′-TTGTGCAGTTCCTGCTGTTGT-3′, and 5′-6FAM-AATGGAACCAAATGGCACCTTCAGCAAT-BHQ1-3′; the primers for GPR109A were from ABI (catalog No. Mm02620500 s1); and the primers for GAPDH were from ABI (catalog No. 4352932E). Quantitative PCR was performed on Stratagene Mx3000P quantitative PCR machines with the Stratagene Brilliant QRT-PCR Master Mix Kit, 1-Step (catalog No. 600551) using 100 ng RNA/well and normalized with mouse GAPDH (ABI).

Inositol phosphate accumulation assay

HEK293 cells were obtained from the American Type Culture Collection. Yttrium silicate scintillation proximity assay beads were obtained from Amersham. Tritiated inositol (50 to 80 Ci/mmol) was obtained from Amersham. Chimeric G proteins, Gα15, Gα16, aequorin, and GPR81, were cloned by PCR using the published sequence data (15) and inserted into the plasmid pcDNA3.1 or pZeo (Gα16). HEK293 cells were dispensed onto a poly-D-lysine tissue culture-treated 96-well plate at a density of 25,000 cells/well. The next day, the cells (~80–90% confluent) were transfected with 100 ng of GPR81 and 20 ng of G protein (5:1 ratio) or control vector containing the aequorin gene using Lipofectamine2000 according to the manufacturer's instructions. Six hours after transfection, the medium was replaced with inositol-free DMEM/10% dialyzed FCS supplemented with 1 μCi/ml tritiated inositol. After incubation overnight, the medium was replaced with HBSS/0.01% BSA containing 10 mM LiCl and incubated at 37°C for 1 h. The medium was aspirated, and the cells were fixed with ice-cold 20 mM formic acid. After incubation at 4°C for 5 h, the lysates were added to yttrium silicate scintillation proximity assay beads, allowed to settle overnight, and read on a Beckman TopCount scintillation counter.

Construction of human CysLT2R/GPR81 chimera and cloning

The extracellular, transmembrane, and intracellular domains for CysLT2R and GPR81 were determined using the TMHMM2.0 software package (Invitrogen, Vector NTI). The chimeric receptor construct consisted of the N terminus, all three extracellular loops, and all seven transmembrane domains from CysLT2R and all three intracellular loops and the C terminus from GPR81. The CysLT2R/GPR81 chimera was synthesized by Blue Heron Biotechnology (Bothell, WA) using GeneMaker technology according to the sequence specified. The chimeric receptor was then amplified using PCR and oligonucleotide primers 5′-CACCATGGAGAGAAAATTTATGTCCTTGC-3′ and 5′-AGCTTCTAGATCAGTGCCACTCAACAATGTGGGGA-3′ and subcloned into pEF6/V5-His-TOPO vector (Invitrogen).

Aequorin assay

CHO cells were transfected with CysLT2R/GPR81 DNA, Aeq DNA, and Gqi9 DNA using Lipofectamine2000 (Invitrogen). Vector plasmid, GPR81, and CysLTR2 DNA were used as controls. A total of 200 ng/ml pertussis toxin (PTX; Calbiochem) was added as indicated to the medium to treat the cells for 18 h. One day after transfection, the cells were resuspended in 20 ml of HBSS buffer containing 2.3 μM coelenterazine F. The cells were labeled for 2 h in the dark with periodic agitation. Aequorin luminescence resulting from intracellular calcium mobilization upon the addition of LTD4 was measured using the microplate luminometer (EG&G Bertholt, Gaithersburg, MD).

Transgenic animal preparations

CysLT2R/GPR81 chimeric receptor was cloned into the *Kpn*I and *Fse*I sites of aP2-pMCS5 transgenic vector (16). The *Sph*I-to-*Asc*I fragment containing the aP2 promoter and the CysLT2R/GPR81 chimeric receptor excised from the vector was gel-purified and injected into the pronuclear embryos of FVB mice. Injected embryos were transferred into the oviducts of pseudopregnant CD1 female mice. Transgenic founder mice were identified by SV40pA sequence-specific PCR genotyping using primers FWD (5′-GATGAGTTTGGACAAACCACA-3′) and REV (5′-CCGGATCATAATCAGCCATAC-3′). One founder, which gave the highest level of chimeric receptor expression, was chosen for all experiments described in this report. All animal experiments were approved by the Institutional Animal Care and Use Committee of Amgen.

Preparation of mouse primary adipocytes and lipolysis assay

Adipocytes were released from epididymal fat pads of male CysLT2R/GPR81 transgenic mice or wild-type mice by collagenase (Sigma catalog No. C2674) digestion similar to previously described methods (3, 17). Cells were then washed four times with KRB buffer (Sigma catalog No. K4002) with 3% fatty acid-free BSA (Sigma) and 1 U/ml adenosine deaminase (Biocatalytics, Inc.; ADA-101). The primary adipocytes harvested from 15 mice were plated onto one 24-well plate and then incubated at 37°C with mild shaking in the presence or absence of LTD4. Nicotinic acid was added as a positive control. Aliquots were collected from the centers of the wells hourly for glycerol assay using Free Glycerol Reagent (Sigma catalog No. F6428).

RESULTS AND DISCUSSIONS

GPR81 is highly expressed in adipose tissue and upregulated during adipocyte differentiation

An earlier report suggests that GPR81 expression is fairly restricted to adipocytes in humans (2). To understand the potential physiological role of GPR81 and to confirm this restricted expression pattern, we analyzed GPR81 expression in a mouse tissue panel using quantitative PCR-based analysis. Our results show that, similar to the report for human tissue samples (2), GPR81 was expressed mainly in the white adipose tissues and that relatively low levels were detected in other tissues (**Fig. 1A**). The expression analysis also revealed that GPR81 expression was markedly induced during the 3T3-L1 preadipocyte differentiation process in vitro (Fig. 1A). Because GPR81 and GPR109A share significant sequence homology, GPR109A expression was also analyzed from the same tissue panel as a comparison. Again, similar to the report for human tissues (2), GPR109A expression was largely restricted to adipose tissue and spleen (Fig. 1B). GPR109A expression was also markedly induced during the 3T3-L1 preadipocyte differentiation process in vitro, although induction appeared earlier than for GPR81 (Fig. 1B).

GPR81 couples to the Gi signaling pathway

Chimeric G proteins are useful reagents for converting signals generated by Gi-coupled receptors to a Gq-mediated signaling pathway (15, 18). This allows for the

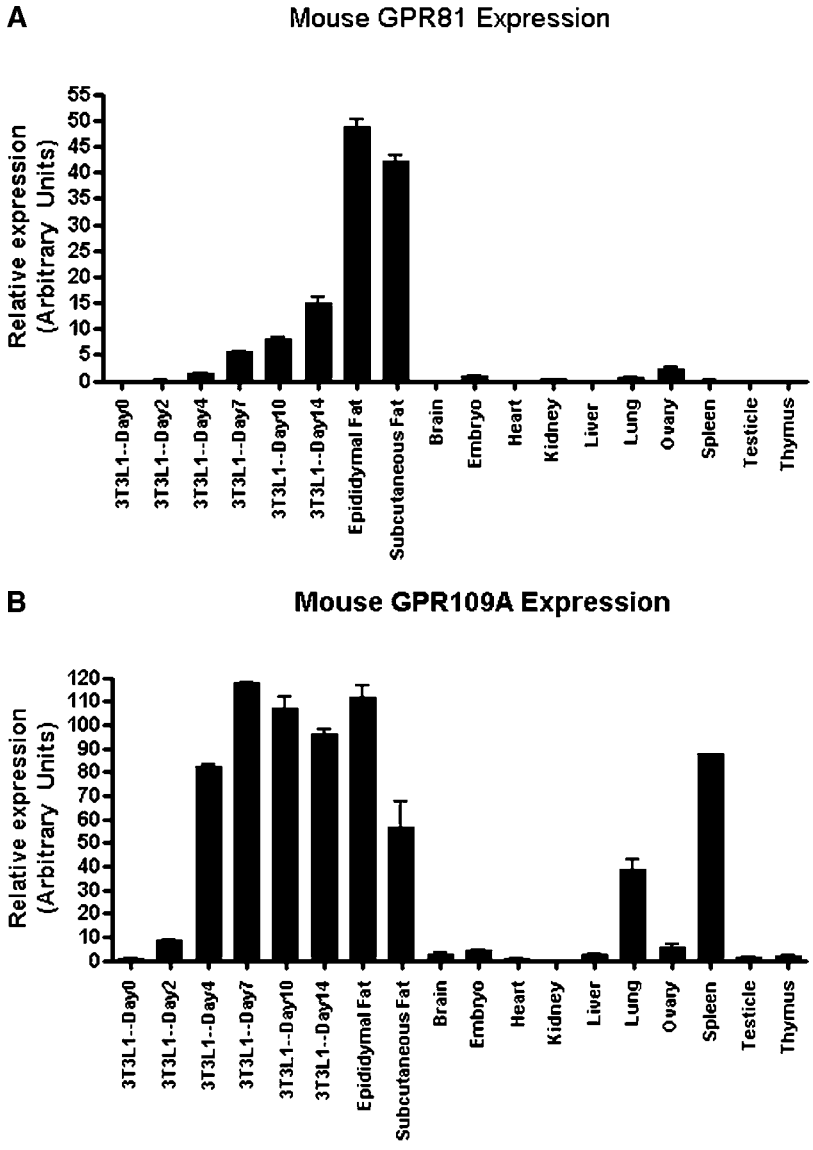
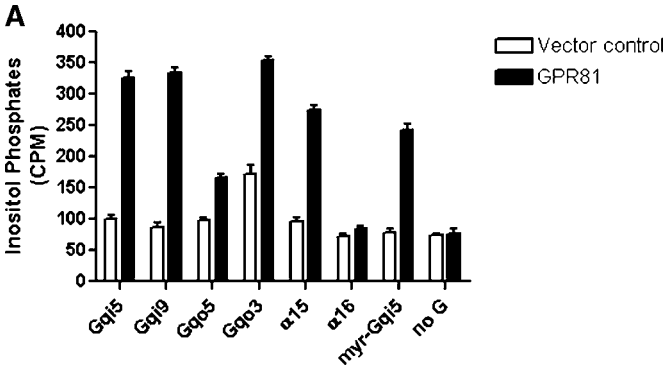


Fig. 1. Distribution of GPR81 transcripts in mouse tissues. Quantitative PCR analyses of mouse tissue samples with mouse GPR81-specific (A) and mouse GPR109A-specific (B) primers. Samples taken at different times during the mouse 3T3-L1 adipocyte differentiation process were also included. Each bar represents the mean value of triplicate data determinations from a single repeated experiment, and results were normalized with mouse GAPDH. Error bars indicate ± SEM.

measurement of Gi-coupled receptor activity in an inositol phosphate (IP) accumulation assay. The IP assay is generally more robust than traditional adenylate cyclase assays (19). The development of chimeric G proteins by Conklin and colleagues (18) has had a significant impact upon GPCR research and on orphan GPCR research in particular.

Shown in **Fig. 2** are the results of transfecting GPR81 into HEK293 cells with or without chimeric G proteins and monitoring of IP accumulation in the presence of 10 mM LiCl to inhibit inositol monophosphatase. In the absence of chimeric G proteins, GPR81 showed no IP accumulation. However, in the presence of Gqi5, Gqi9, myristolyated Gqi5, and Gα15, robust constitutive activity was observed compared with the vector control. Gqi9 showed the highest level of activity, which was ~4.2-fold greater than the basal level of vector/Gqi9. Myristolyated Gqi5 showed the next highest level of constitutive activity (3.6-fold), although this was not significantly different from that of Gqi9. Gqo5 and Gqo3 also demonstrated constitutive activity to a lesser degree. The basal level of Gqo3 was approximately twice that of the other chimeric G proteins. This is perhaps attributable to the ability of Gqo3 to couple unidentified endogenous Gi-coupled receptors present in HEK293 cells. Interestingly, the human homolog of Gα15, Gα16, showed no activity (20, 21). These results strongly suggest that GPR81 couples to the Gi signaling pathway in HEK293 cells. They are also consistent with data showing that the nicotinic acid receptor, which is closely related to the GPR81, is also coupled to the Gi signaling pathway, based upon niacin's ability to inhibit forskolin-induced cAMP accumulation (6). Other Gi-coupled receptors have been shown to couple to chimeric G proteins with a comparable level of activity as GPR81 (22).



B

G-Protein	Fold over Basal	S.D. (n=3)
Gqi5	3.1	0.4
Gqi9	4.2	0.4
Gqo5	1.8	0.1
Gqo3	1.9	0.1
alpha15	3.0	0.4
alpha16	1.2	0.1
myrGqi5	3.6	0.4
none	1.0	

Fig. 2. Constitutive activity of GPR81 in an inositol phosphate (IP) accumulation assay. HEK293 cells were transiently transfected with GPR81 alone (no G) or in combination with various chimeric G proteins, Gα15, or Gα16. Control vector containing aequorin cDNA served as a negative control. Twenty-four hours after transfection, the cells were incubated with HBSS containing 10 mM LiCl to initiate IP accumulation and measure constitutive activity. A: Data are representative of three experiments. Results are presented as means of quadruplicate determinations ± SD. B: Data from three experiments are averaged and expressed as fold over basal.

Construction and characterization of a chimeric receptor between CysLT2R and GPR81

Because GPR81 is an orphan receptor, it is difficult to study the receptor signaling and function without access to natural or synthetic agonists. To provide more direct evidence that GPR81 couples to the Gi pathway and to study the potential effects of the receptor activation on adipocyte functions, we generated a chimeric receptor between cysteinyl leukotriene receptor, CysLT2R (23, 24), and GPR81. The chimeric receptor was designed to contain the three intracellular loops and the C terminus of GPR81, with the expectation that the hybrid receptor signaling would resemble that of native GPR81. The chimeric receptor also contained extracellular and transmembrane domains from CysLT2R to allow the response of the chimeric receptor to CysLT2R ligand, LTD4 (**Fig. 3A**). This strategy has worked well for several other GPCRs (25–30).

We next studied the signaling pathways that are activated by the CysLT2R/GPR81 chimeric receptor in response to LTD4 treatment. No activation was observed through the Gs and Gq pathways (data not shown). However, when CysLT2R/GPR81 was cotransfected with Gqi9 into CHO cells, intracellular calcium mobilization occurred in response to LTD4 treatment, and this signaling was PTX-sensitive (Fig. 3B). The wild-type GPR81 receptor did not respond to LTD4 treatment in this assay format (Fig. 3B). As a control for these experiments, the native CysLT2R, when transfected into CHO cells, responded to LTD4 treatment as described previously, and this response was PTX-insensitive, as the native CysLT2R coupled to the Gq signaling pathway (Fig. 3C) (23, 24). These results demonstrate that the chimeric receptor signals through Gi pathways. Because the intracellular loops and the C-terminal domain of the chimeric receptor are derived from GPR81, these results provided additional evidence that GPR81 is able to signal through Gi proteins for receptor activation.

Activation of the CysLT2R/GPR81 chimeric receptor by LTD4 inhibits lipolysis in mouse adipocytes

To study the potential effects of GPR81 activation on adipocyte function, transgenic mice overexpressing CysLT2R/GPR81 under of the control of a fat-specific promoter, aP2, were generated. Transgenic animals appeared normal with no apparent abnormalities. Furthermore, there was no difference in body weight or plasma free fatty acid levels in transgenic versus wild-type littermates (data

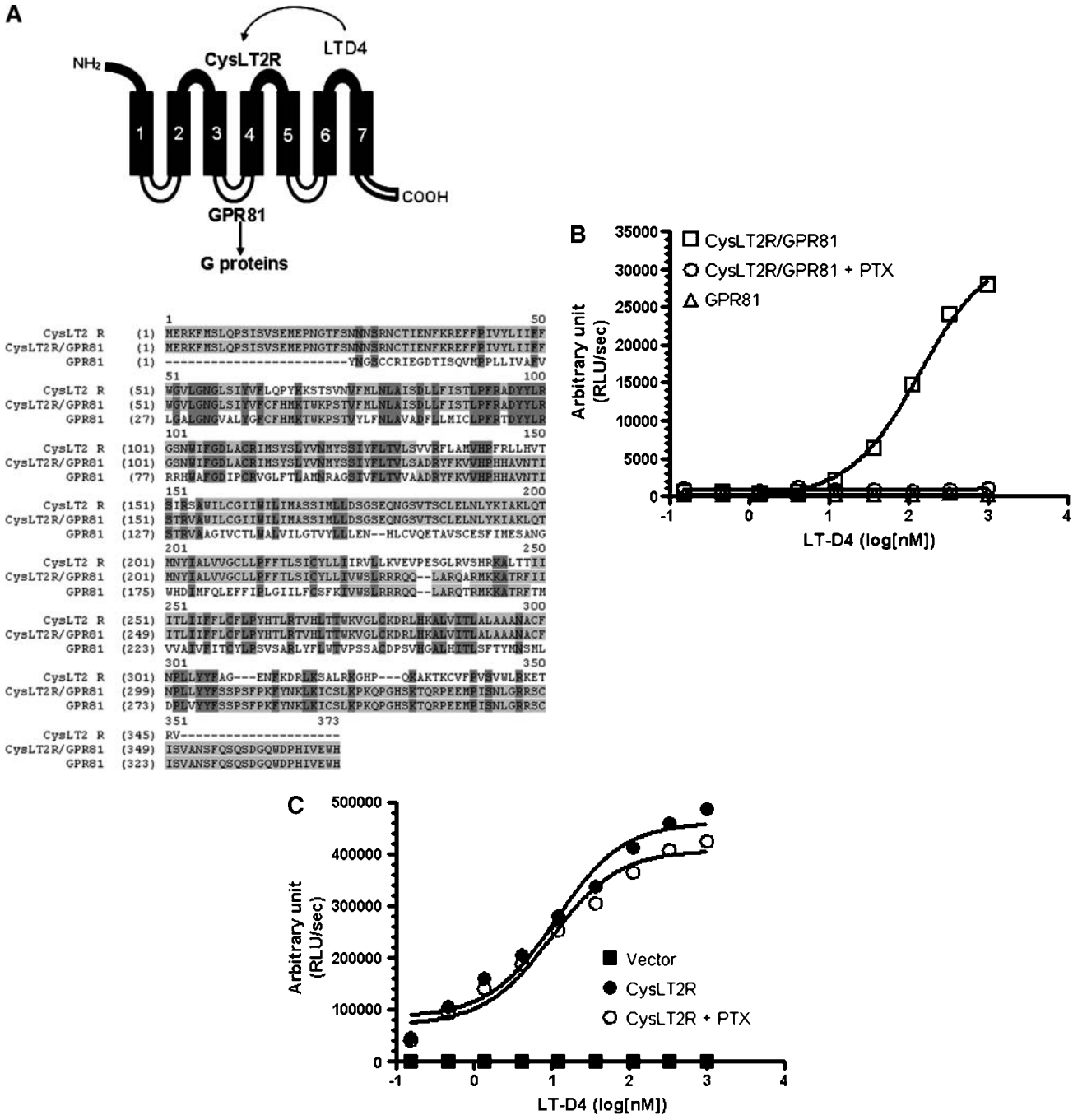


Fig. 3. Construction and signaling of cysteinyl leukotriene 2 receptor (CysLT2R)/GPR81 chimeric receptor. A: Amino acid sequence alignment of CysLT2R, GPR81, and chimeric CysLT2R/GPR81 receptors generated by the Vector NTI program (Invitrogen). B, C: CHO cells were transiently transfected with vectors containing various receptors, chimeric G protein-Gqi9, and aequorin. Calcium flux was converted and measured as luminescent signals from aequorin. RLU, relative light units; PTX, pertussis toxin.

not shown). The relative expression levels of endogenous GPR81 and CysLT2R/GPR81 chimeric receptors in the wild-type and transgenic animals were examined. As shown in **Fig. 4A**, chimeric receptor expression was detected only in the transgenic animals and not in the wild-type littermates. The effect of transgene on endogenous GPR81 expression was also examined, and a small increase was observed (Fig. 4B). To examine whether the chimeric receptor could affect lipolysis in adipocytes, glycerol release was measured after LTD4 treatment in primary adipocytes isolated from either wild-type or CysLT2R/GPR81 transgenic mice. Nicotinic acid was used as a positive control in these experiments. As shown in Fig. 4C, D, nicotinic acid treatment resulted in the inhibition of lipolysis in both wild-type and CysLT2R/GPR81-expressing adipocytes to a similar extent in a time-dependent manner. In contrast,

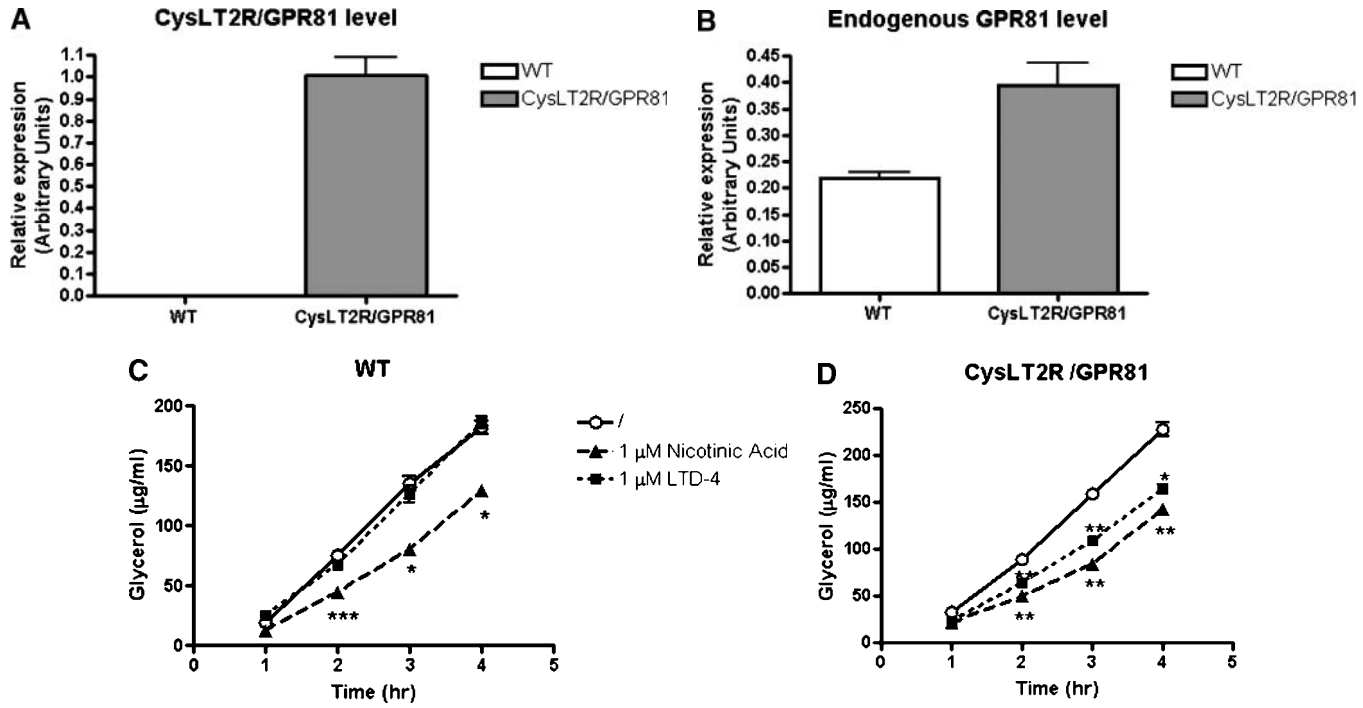


Fig. 4. Leukotriene D_4 (LTD4) inhibits lipolysis in isolated adipocytes expressing CysLT2R/GPR81 chimeric receptor. A, B: Expression levels of CysLT2R/GPR81 chimeric receptor (A) and endogenous GPR81 (B) in the fat of wild-type (WT) or transgenic mice were determined by quantitative PCR analysis and normalized to mouse GAPDH. Results are averages of triplicate data points from three different animals from each group. C, D: Isolated adipocytes from wild-type (C) or CysLT2R/GPR81 transgenic (D) mice were treated with buffer control, LTD4, or nicotinic acid. Glycerol release was measured hourly in the medium using Free Glycerol Reagent. Data are representative of three experiments. Results are presented as means of duplicate determinations. Error bars indicate ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

LTD4 treatment only inhibited lipolysis in adipocytes isolated from CysLT2R/GPR81 transgenic animals but not in cells from wild-type animals. The extent of antilipolytic effects was similar between LTD4 and nicotinic acid treatments (Fig. 4C, D). These results suggest that the activation of native GPR81 receptor in adipocytes may also lead to the inhibition of lipolysis.

Obesity is commonly associated with insulin resistance, hyperinsulinemia, and dyslipidemia, which are all important cardiovascular risk factors. The identification of novel, safe, and effective therapies for cardiovascular diseases has been the subject of intense pharmaceutical/biotech research efforts. Nicotinic acid still remains the most effective therapy for increased HDL while decreasing other cardiovascular risk factors, including VLDL, LDL, lipoprotein [a], and triglycerides. However, the therapeutic value of nicotinic acid is limited by its major side effect of cutaneous flushing, which is mediated by the expression of GPR109A in immune cells (6). Clearly, there is much room for other novel effective therapies with more manageable side effect profiles.

In this report, we examined the potential signaling pathways and functions of adipocyte-expressed orphan GPR81. Results from constitutive activities observed in the IP accumulation assay using chimeric G proteins that convert Gi signaling to Gq signaling, as well as Gi pathway activation from a chimeric receptor between CysLT2R and GPR81, argue that GPR81 couples to the Gi family of proteins. These findings, together with the well-known effects of antilipolytic activities of Gi activation, suggest that GPR81 may regulate this process in adipocytes as well. Indeed, the activation of adipocytes isolated from transgenic animals expressing the CysLT2R/GPR81 chimeric receptor by LTD4 results in the inhibition of lipolysis in vitro. Although our understanding of the lipid-lowering profile of nicotinic acid is far from complete, it has been proposed that the mechanism of action involves the inhibition of adipocyte lipolysis via activation of the Gi pathway. Given that GPR81 likely couples to the Gi signaling pathway and that its expression is more restricted to adipocytes than GPR109A, we speculate that the activation of GPR81 has potential utility in treating dyslipidemia without the flushing side effect observed with nicotinic acid treatment. Identification of GPR81 ligands could provide better understanding of its functions in adipocyte biology and its potential utility in treating metabolic diseases.

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