Free fatty acids (FFAs) play important physiological roles in many tissues as an energy source and as signaling molecules in various cellular processes. Elevated levels of circulating FFAs are associated with obesity, dyslipidemia, and diabetes. Here we show that GPR84, a previously orphan G protein-coupled receptor, functions as a receptor for medium-chain FFAs with carbon chain lengths of 9–14. Medium-chain FFAs elicit calcium mobilization, inhibit 3′,5′-cyclic AMP production, and stimulate [35S]guanosine 5′-O-(3-thiotriphosphate) binding in a GPR84-dependent manner. The activation of GPR84 by medium-chain FFAs couples primarily to a pertussis toxin-sensitive Gi/o pathway. In addition, we show that GPR84 is selectively expressed in leukocytes and markedly induced in monocytes/macrophages upon activation by lipopolysaccharide. Furthermore, we demonstrate that medium-chain FFAs amplify lipopolysaccharide-stimulated production of the proinflammatory cytokine interleukin-12 p40 through GPR84. Our results indicate a role for GPR84 in directly linking fatty acid metabolism to immunological regulation.

G protein-coupled receptors (GPCRs) constitute one of the largest gene families yet identified (1, 2). It has been estimated that more than half of all modern drugs target these receptors (3, 4). GPCRs contain seven transmembrane domains and are activated by a wide variety of ligand types, including light, ions, amino acids, nucleotides, lipids, peptides, and proteins. In addition to about 250 characterized receptors, over 100 human genes encode proteins that belong to this family of receptors but for which ligands and functions remain to be determined (1). These orphan receptors are expected to play important roles in the regulation of a diversity of physiological functions.

In the past decade an increasing number of GPCRs have been deorphanized. Many of the identified ligands are metabolic intermediates, including succinate (ligand for GPR91) (5), α-ketoglutarate (ligand for GPR99) (5), fatty acids (ligands for GPR40/41/43/120) (6–10), ketone body (ligand for HM74a) (11), bile acids (ligands for BG37) (12), and kynurenine acid (ligand for GPR35) (13). We have built a library of biochemical intermediates to test their ability to activate orphan GPCRs. In this report, we have identified medium-chain FFAs as ligands for GPR84. Short-chain and long-chain saturated and unsaturated FFAs, previously shown to activate GPR40/41/43/120 (6–10), are inactive at GPR84. GPR84 is an orphan GPCR originally isolated using an expressed sequence tag data mining strategy (14) and as a gene differentially expressed in granulocytes (15). No close homologs of GPR84 could be identified, although GPR84 is distantly related to monoamine receptors. Expression analysis revealed significant induction of GPR84 in monocytes/macrophages upon lipopolysaccharide (LPS) stimulation, suggesting that medium-chain FFAs may regulate inflammatory responses through activation of GPR84.

**EXPERIMENTAL PROCEDURES**

*Cloning and Cell Culture—* Human and mouse GPR84 were cloned by PCR from human and mouse bone marrow cDNA (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. Purified human monocytes (AllCells) and THP-1 and RAW264.7 cells (ATCC) were incubated with 100 ng/ml LPS (Escherichia coli 0111:B4) before harvesting for RNA preparation. All cells were cultured at 37 °C with 5% CO2. CHO-GPR84 stable cells were generated by transfecting CHO cells with N-terminal-FLAG-tagged human GPR84 and subsequently were selected in 1 mg/ml G418 (Cellgro). All compounds used in this study were purchased from Sigma.

*Aequorin Assay—* CHO cells were transiently transfected with GPR84 and aequorin reporter (Euroscreen) using Lipofectamine 2000 reagent (Invitrogen) (5, 16). Expression of the reporter was measured as the luminescence produced by the calcium-sensitive aequorin reporter using a luminometer. Aequorin luminescence was excited with 425 nm light and recorded over a period of 20 s with a MicroLumat luminometer (Berthold).

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3 The abbreviations used are: GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-O-(3-thiotriphosphate); [Ca2+]i, intracellular calcium concentration; EC50, medium effective concentration; CHO, Chinese hamster ovary; LPS, lipopolysaccharide; FFA, free fatty acid; FAM, 6-carboxyfluorescein; BHQ, black hole quencher; Q-RT-PCR, quantitative reverse transcriptase-mediated PCR; IL, interleukin; Th, T helper.
Immunofluorescence Staining—Cells were fixed with 4% paraformaldehyde, blocked with 5% goat serum in phosphate-buffered saline, and incubated with anti-FLAG M2 monoclonal antibody (Sigma) for 1 h on ice. After extensive washing in phosphate-buffered saline, cells were incubated with goat anti-mouse IgG-rhodamine secondary antibody for an additional 30 min. Images were captured with a charge-coupled device digital camera connected to a Leica DC500 microscope.

Flow Cytometry Analysis—CHO-GPR84 and CHO-Vector cells were stained with anti-FLAG M2 monoclonal antibody in phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide for 1 h at 4 °C. After extensive washing, cells were incubated with goat anti-mouse IgG-fluorescein isothiocyanate secondary antibody (Caltag). Flow cytometry analysis was carried out using FACS Calibur (BD Biosciences).

Cyclic AMP Assay—CHO-GPR84 and CHO-Vector cells were seeded at 2 × 10^4 cells/well in 96-well plates. Cells were stimulated in Opti-MEM (Invitrogen) with compounds for 20 min before treatment with 10 μM forskolin for a further 20 min at 37 °C. A cyclic AMP (cAMP) assay was performed with the cAMP-Screen system (Applied Biosystems). When indicated, 100 ng/ml pertussis toxin (Calbiochem) was incubated with the cells for 16 h.

GTP\_S Binding Assay—CHO-GPR84 and CHO-Vector cells were 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 resuspending the pellet in 20 mM HEPES (pH 7.5), 5 mM MgCl₂, 25 μg of membranes was incubated at room temperature for 1 h in assay buffer (20 mM HEPES, 5 mM MgCl₂, 160 mM NaCl, 0.05% bovine serum albumin, pH 7.5) containing 10 μM GDP and 0.1 nM [35S]GTP\_S (PerkinElmer Life Sciences) in the absence or presence of compounds. Reactions were terminated by vacuum filtration through GF/B filters, and the retained radioactivity was quantified on a liquid scintillation counter.

Quantitative Reverse Transcriptase-mediated PCR (Q-RT-PCR) Analysis—Total RNA from human or mouse tissues (Clontech) and human immune cells (AllCells, LLC) were
treated with DNase I (Ambion) before reverse transcription. Q-RT-PCR was performed on an ABI Prism 7700 sequence detector using TaqMan PCR core reagents (Applied Biosystems). Ratios of GPR84 to glyceraldehyde-3-phosphate dehydrogenase message RNA were calculated using a \( \Delta\Delta CT \) method (Applied Biosystems). Primers and probes were designed using

FIGURE 3. Effect on intracellular cAMP accumulation. The data represent the means ± S.E. for triplicate determinations. A, inhibition of cAMP production by medium-chain fatty acids in GPR84-expressing cells. CHO-GPR84 cells or CHO-Vector cells were incubated with nonanoic acid, capric acid, undecanoic acid, or lauric acid for 20 min before addition of 10 \( \mu \text{M} \) forskolin. B, effect of pertussis toxin (PTX) on cAMP production. Cells were pretreated with 100 ng/ml pertussis toxin for 16 h when indicated.

FIGURE 4. Effect on \( {^{35}}\text{S} \text{GTP}\gamma\text{S} \) binding. Data are shown as means ± S.E. for triplicate determinations. A, induction of \( {^{35}}\text{S} \text{GTP}\gamma\text{S} \) binding by medium-chain fatty acids. Membranes from CHO-GPR84 cells or CHO-Vector cells were incubated with capric acid, undecanoic acid, or lauric acid for 1 h at room temperature. B, effect of pertussis toxin (PTX) on \( {^{35}}\text{S} \text{GTP}\gamma\text{S} \) binding. Pertussis toxin pretreatment abolished GPR84 activation by medium-chain fatty acids. C, effect of short-chain fatty acid acetate and long-chain fatty acid docosahexaenoic acid (DHA) in the \( {^{35}}\text{S} \text{GTP}\gamma\text{S} \) binding assay.
Medium-chain Fatty Acids as Ligands for GPR84

Primer Express software (Applied Biosystems). The primer and probe sequences for human GPR84 were: forward, TTCAGC- CCTCTCTGTTGGACA; reverse, TGCAGAAGTGGCAG- CCG; probe, 5’-FAM-CTACCTCCACCTGCACTGGCGCA- BHQ-3’. Primer and probe sequences for mouse GPR84 were: forward, GACAAATCGGCTGCTACAGG; reverse, CAG- GACATGCTTCTTGTG; probe, 5’-FAM-AGACCTGCT- CCTACAGGTTGCT-BHQ-3’. Primer and probe sequences for mouse interleukin-12 p40 subunit were: forward, AAGCCCT- GCCGATTGAACCT; reverse, GAAGCTGGTGCTGTAGTTT- TCATATT; probe, 5’-FAM-CGTTGGAAGGCAGCCGCA- GAA-BHQ-3’.

Cytokine Secretion—RAW264.7 cells were seeded at a density of 5 × 10⁴ cells/well in 96-well plates in Dulbecco’s modified Eagle’s medium containing 0.25% (v/v) fetal bovine serum. Compounds were added 1 h before LPS (final concentration 100 ng/ml). Cells were incubated at 37 °C for 21 h, and supernatants were collected for cytokine assay. Untreated cells were used as controls. Cytokine concentrations were determined using the Bio-Plex cytokine assay system (Bio-Rad) following the manufacturer’s instructions. Data were analyzed with Bio-Plex Manager 2.0 software with 4- and 5-parameter curve fitting.

RESULTS

To search for natural ligands for orphan GPCRs, we tested a collection of biochemical intermediates for their ability to evoke an increase in intracellular Ca²⁺ concentration ([Ca²⁺]i) using the aequorin assay (16). CHO cells were transiently transfected with plasmids encoding human GPR84, aequorin reporter, and a mixture of small G proteins (Gq, Gs, and Gqi9). These promiscuous and chimeric small G proteins have been reported to convert the signaling pathways of non- Gq-coupled GPCRs to calcium mobilization (18–20). Caprylic acid (C8:0) evoked an increase of [Ca²⁺]i with a medium effective concentration (EC₅₀) of 48 μM, in cells expressing human GPR84 (Fig. 1A). A similar response was observed for undecanoic acid (C11:0) (Fig. 1A). Control cells did not respond to caprylic acid or undecanoic acid (Fig. 1A). Neither caprylic acid nor undecanoic acid activated any of ~40 other GPCRs (data not shown).

To investigate the G protein specificity of GPR84, CHO cells were transfected with plasmids encoding human GPR84 and individual small G proteins and tested in aequorin assay. The chimeric G protein Gqi9 significantly potentiated the activation of GPR84 by caprylic acid, whereas the Gq5 chimera did not (Fig. 1B), suggesting that GPR84 may signal through a Gq/i/o pathway.

The murine ortholog of GPR84 shares 85% amino acid identity with human GPR84. Similarly, medium-chain FFAs (caprylic acid, undecanoic acid, and lauric acid) activated murine GPR84 in the presence of Gqi9 chimera (Fig. 1C).

CHO cells stably expressing N-terminally FLAG-tagged human GPR84 (CHO-GPR84) were generated. Immunofluorescence staining of nonpermeabilized CHO-GPR84 cells revealed prominent plasma membrane localization of GPR84 protein (Fig. 2A). The cell surface expression of GPR84 was further confirmed by flow cytometry (Fig. 2B).

| FFA                        | cAMP assay | [35S]GTPγS binding assay |
|----------------------------|------------|--------------------------|
|                           | μM         |                          |
| Formic acid (C1:0)         | inactive   | inactive                 |
| Acetic acid (C2:0)         | inactive   | inactive                 |
| Propionic acid (C3:0)      | inactive   | inactive                 |
| Butyric acid (C4:0)        | inactive   | inactive                 |
| Caprylic acid (C6:0)       | inactive   | inactive                 |
| Heptanoic acid (C7:0)      | inactive   | inactive                 |
| Caprylic acid (C8:0)       | inactive   | >100                     |
| Nonanoic acid (C9:0)       | 52.3 ± 5.6 | 70.2 ± 2.2               |
| Capric acid (C10:0)        | 4.5 ± 0.3  | 4.6 ± 0.1                |
| Undecanoic acid (C11:0)    | 7.7 ± 0.1  | 8.6 ± 0.7                |
| Lauric acid (C12:0)        | 8.8 ± 0.2  | 10.5 ± 0.2               |
| Tridecanoic acid (C13:0)   | 24.8 ± 1.1 | 21.4 ± 1.3               |
| Myristic acid (C14:0)      | 93.2 ± 11.0| 14.4 ± 5.4               |
| Palmitic acid (C16:0)      | inactive   | inactive                 |
| Palmitoleic acid (C16:1)   | inactive   | inactive                 |
| Oleic acid (C18:1)         | inactive   | inactive                 |
| Elaidic acid (C18:1)       | inactive   | inactive                 |
| Linoleic acid (C18:2)      | inactive   | inactive                 |
| α-Linolenic acid (C18:3)   | inactive   | inactive                 |
| γ-Linolenic acid (C18:3)   | inactive   | inactive                 |
| cis-11,14,17-Eicosatrienoic acid (C20:3) | inactive | |
| Arachidonic acid (C20:4)   | inactive   | inactive                 |
| cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5) | inactive | |
| Docosahexaenoic acid (C22:6) | inactive | |

Medium-chain FFAs inhibited forskolin-stimulated 3',5'-cAMP production dose-dependently in CHO-GPR84 cells (Fig. 3A). The EC₅₀ values for nonanoic acid, caprylic acid, undecanoic acid, and lauric acid were 52, 4, 8, and 9 μM, respectively. FFAs had no effects on cAMP concentrations in vector-transfected cells (Fig. 3A). FFA-induced inhibition of cAMP production was blocked completely by preincubating cells with pertussis toxin, a specific inhibitor of Gqi/o proteins (Fig. 3B).

In addition, medium-chain FFAs stimulated [35S]GTPγS incorporation in membranes prepared from CHO-GPR84 cells (Fig. 4A), an effect that was also abolished by pertussis toxin treatment (Fig. 4B). In the [35S]GTPγS binding assay, the EC₅₀ values for caprylic acid, undecanoic acid, and lauric acid were 5, 9, and 10 μM, respectively. The short-chain FFA acetic acid and long-chain FFA docosahexaenoic acid were inactive at GPR84 (Fig. 4C). Similar results were obtained in cAMP and [35S]GTPγS assays using independent CHO clones stably expressing GPR84 (data not shown).

Medium-chain FFAs were unable to promote cAMP production in CHO-GPR84 cells in the absence of forskolin, suggesting that GPR84 does not signal through a Gs-mediated pathway (data not shown). GPR84 is unlikely to couple to a Gs pathway, because medium-chain FFAs did not induce calcium mobilization in the absence of co-transfected chimeric G proteins (Fig. 1B), nor did they stimulate inositol phosphate accumulation in cells expressing GPR4 (data not shown). Taken together, our
results suggest that GPR84 activation by medium-chain FFAs couples primarily to a pertussis toxin-sensitive Gi/o pathway. The potency of various FFAs at GPR84 examined in the cAMP and [35S]GTPγS binding assays are summarized in Table 1. Importantly, we found no evidence of GPR84 activation by short-chain FFAs, long-chain saturated FFAs, or long-chain unsaturated FFAs (Table 1). Our studies demonstrate that GPR84 functions as a specific receptor for medium-chain FFAs of C9 to C14 in length, with C10−C12 being the most potent.

Expression analysis by Q-RT-PCR revealed that the messenger RNAs of both human and mouse GPR84 are expressed predominantly in hematopoietic tissues (Fig. 5). In human, high levels of GPR84 expression were detected in the bone marrow and, to a lesser extent, in the peripheral leukocytes and the lung (Fig. 5A). In mouse, GPR84 mRNA was expressed mainly in the bone marrow, with transcripts also detected in the spleen, the lymph node, and the lung (Fig. 5B). The expression profile of GPR84 is notably different from that of GPR40, a pancreatic islet-specific GPCR activated by medium- and long-chain FFAs (6, 9).

Among various subpopulations of leukocytes tested, GPR84 was detected primarily in monocytes and neutrophils (Fig. 6A). Additionally, GPR84 expression was increased in purified human monocytes upon LPS activation (Fig. 6B). Moreover, GPR84 mRNA levels were markedly up-regulated by LPS treatment in the human monocytic leukemia cell line THP-1 and the mouse monocyte/macrophage cell line RAW264.7 (Fig. 6, C and D), suggesting that GPR84 may play a pivotal role in monocyte/macrophage activation and host immune response.

A small molecule GPR84 surrogate agonist (21), diindolylmethane, activated GPR84 with greater potency than the natural ligand capric acid (Fig. 7). The EC50 values for diindolylmethane in the cAMP and [35S]GTPγS binding assays were 0.7 and 0.5 μM, respectively, versus 4.5 and 4.6 μM for capric acid. Diindolylmethane was therefore used as a tool compound, along with medium-chain FFAs, to investigate the effect of GPR84 activation on cytokine secretion.

Capric acid, undecanoic acid, and lauric acid dose-dependently increased the secretion of interleukin-12 p40 subunit (IL-12 p40) from LPS-stimulated RAW264.7 cells (Fig. 8A). Similar results were observed with diindolylmethane, suggesting that this effect is mediated through GPR84 activation (Fig. 8A). The up-regulation of IL-12 p40 mRNA by GPR84 agonists was further confirmed by Q-RT-PCR analysis (Fig. 8B). No effect on IL-12 p40 was observed in the absence of LPS (Fig. 8C). Among more than 20 cytokines analyzed (data not shown), IL-12 p40 was uniquely induced by GPR84 agonists (capric acid, undecanoic acid, lauric acid, and diindolylmethane) in the presence of LPS. The short-chain FFAs (acetic acid and propionic acid) and long-chain FFA (docosahexaenoic acid) exhibited no stimulatory activity (Fig. 8). Taken together, our results indicate that medium-chain FFAs amplify LPS-stimulated production of IL-12 p40 through GPR84.
DISCUSSION

In the current study, we have shown that the previously orphan GPCR, GPR84, functions as a specific receptor for medium-chain FFAs of C₉ to C₁₄ length, with capric acid (C₁₀:0), undecanoic acid (C₁₁:0), and lauric acid (C₁₂:0) being the most potent agonists. Medium-chain FFAs activated GPR84 in aequorin assays in the presence of Gₛᵣ chimeric G proteins. Medium-chain FFAs inhibited forskolin-induced cAMP production and stimulated [³⁵S]GTPγS binding in a GPR84-dependent manner. Our results also suggest that GPR84 activation by medium-chain FFAs is coupled to a pertussis toxin-sensitive Gᵢ/ᵦ pathway.

In the past few years, several GPCRs have been identified as receptors for FFAs. GPR41 and GPR43 can be specifically activated by short-chain FFAs (7, 8). GPR40 mediates FFA-induced insulin secretion from pancreatic β cells as a receptor for medium- and long-chain FFAs (6, 9). GPR120, a receptor for long-chain unsaturated FFAs, is responsible for FFA-induced glucagon-like peptide-1 secretion from intestinal neuroendocrine L-cells (10, 22). The identification of GPR84 as an immune cell-specific receptor for FFAs adds to the repertoire of GPCRs that can be activated by FFAs, strengthening the importance of FFAs as signaling molecules in regulating diverse pathophysiological functions.

The plasma concentration of FFAs in the postabsorptive state is ~0.5 mM and can be substantially increased after ingestion of a fatty meal (23). Most of the circulating FFAs are bound with serum albumin such that the concentration of unbound FFAs is in the micromolar range (24). The EC₅₀ values for medium-chain FFAs capric acid, undecanoic acid, and lauric acid at GPR84 (4, 8, and 9 μM, respectively, in the cAMP assay) are then within range of their physiological concentrations and are comparable with their potencies at GPR40 (43 and 6 μM for capric acid and lauric acid, respectively) (6, 9).

Although both can be activated by medium-chain FFAs, GPR84 and GPR40 exhibit clear differences in ligand preference, G protein coupling, and expression profiles. GPR84 is activated by medium-chain FFAs of C₉ to C₁₄ in length, with C₁₀:0, C₁₁:0, and C₁₂:0 being the most potent. Short-chain FFAs and long-chain FFAs are inactive at GPR84. In contrast, GPR40 is promiscuously activated by medium- and long-chain saturated and unsaturated FFAs (6, 9). GPR84 and GPR40 also utilize different intracellular signaling mechanisms. GPR84 couples almost exclusively to pertussis toxin-sensitive Gᵢ/ᵦ pro-
teins, whereas GPR40 activates the G\textsubscript{q} pathway (6, 9). GPR40 is selectively expressed in activated monocytes/macrophages and neutrophils. On the contrary, the highest levels of GPR40 are found in the insulin-producing pancreatic cells, with no or very low expression detected in immune cells (6, 9). The differential localization of GPR84 and GPR40 suggests that they respond to FFAs in a tissue-specific manner and mediate distinct effects of FFAs.

FFAs are known to exert diverse actions on various tissues (25–29). Elevated levels of circulating FFAs are associated with obesity, dyslipidemia and diabetes (30). Although an increased secretion of proinflammatory cytokines and infiltration of monocytes/macrophages into the adipose tissues have been reported in obese patients (31, 32), the mechanism by which FFAs exert their effects on immune cells has not been precisely defined. The specific and abundant expression of GPR84 in monocytes/macrophages and neutrophils suggests a role of medium-chain FFAs in modulating leukocyte functions and host defense, providing a potential link between the obesity-related metabolic syndrome and the proinflammatory state with which it is closely associated.

The proinflammatory cytokine IL-12 plays a pivotal role in promoting cell-mediated immunity to eradicate pathogens by inducing and maintaining T helper 1 (Th1) responses and inhibiting T helper 2 (Th2) responses (33–35). FFAs may affect pathogen elimination processes by inducing IL-12 p40 through GPR84. Moreover, IL-12-driven Th1 reactions have deleterious consequences in autoimmune and inflammatory diseases including multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis. Medium-chain FFAs, through their direct actions on GPR84, may affect Th1/Th2 balance and could provide a link between metabolic disorders and autoimmune diseases.

Interestingly, consistent with IL-12 p40 induction by GPR84, GPR84-deficient T cells exhibited increased production of Th2 cytokines (36). It remains to be determined whether the functions of monocytes/macrophages and neutrophils, the primary sites of GPR84 expression, are altered in a GPR84-deficient
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state, particularly in response to medium-chain FFAs. GPR84-deficient mice will be valuable for dissecting the signaling functions of FFAs.

In summary, we have identified medium-chain FFAs as ligands for the orphan receptor GPR84. We have also shown that GPR84 is highly expressed in immune cells and markedly induced in monocytes/macrophages by LPS. In addition, the activation of GPR84 in monocytes/macrophages amplifies LPS-stimulated IL-12 p40 production. The identification of GPR84 as a leukocyte-specific receptor for FFAs provides a novel mechanism directly linking FFAs metabolism to innate and adaptive immunity, highlighting the importance of FFAs as signaling molecules in regulating a myriad of biological processes.

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