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ORIGINAL ARTICLE

Novel selective ligands for free fatty acid receptors GPR120 and GPR40

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Abstract GPR120 and GPR40 are G-protein-coupled receptors whose endogenous ligands are medium- and long-chain free fatty acids, and they are thought to play an important physiological role in insulin release. Despite recent progress in understanding their roles, much still remains unclear about their pharmacology, and few specific ligands for GPR120 and GPR40 besides medium- to long-chain fatty acids have been reported so far. To identify new selective ligands for these receptors, more than 80 natural compounds were screened, together with a reference compound MEDICA16, which is known to activate GPR40, by monitoring the extracellular regulated kinase (ERK) and $[Ca^{2+}]_i$ responses in inducible and stable expression cell lines for GPR40 and GPR120, respectively. MEDICA16 selectively activated $[Ca^{2+}]_i$ response in GPR40-expressing cells but not in GPR120-expressing cells. Among the natural compounds tested, grifolin derivatives, grifolic acid and grifolic acid methyl ether, promoted ERK and $[Ca^{2+}]_i$ responses in GPR120-expressing cells, but not in GPR40-expressing cells, and inhibited the α-linolenic acid (LA)-induced ERK and $[Ca^{2+}]_i$ responses in GPR120-expressing cells. Interestingly, in accordance with the pharmacological profiles of these compounds, similar profiles of glucagon-like peptide-1 secretion were seen for mouse enteroendocrine cell line, STC-1 cells, which express GPR120 endogenously. Taken together, these studies identified a selective GPR40 agonist and several GPR120 partial agonists. These compounds would be useful probes to further investigate the physiological and pharmacological functions of GPR40 and GPR120.

Keywords GPCR · GPR120 · GPR40 · Free fatty acid receptor · Grifolin · GLP-1

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Introduction

Free fatty acids (FFAs) are not only essential nutritional components, but they also function as signaling molecules. Recently, a G-protein-coupled receptor de-orphanizing strategy successfully identified multiple receptors for FFAs, which function on the cell surface and play significant roles in nutritional regulation. One of these receptors, GPR40, is activated by medium- to long-chain fatty acids (Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003; Hara et al. 2009) and is coupled to G_q, which results in activation of phospholipase C (Hardy et al. 2005) and subsequent increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) (Itoh et al. 2003). In addition, GPR40 has been reported to promote the phosphorylation of extracellular regulated kinase (ERK)-1/2 (Yonezawa et al. 2004). A number of in vitro and in vivo studies have demonstrated that FFAs promote glucose-stimulated insulin secretion in pancreatic β cells via GPR40 (Briscoe et al. 2003; Itoh et al. 2003; Poitout 2003; Steneberg et al. 2005; Feng et al. 2006). GPR120, which is expressed in the intestinal tract and in adipocytes, is activated by medium- to long-chain fatty acids (Hirasawa et al. 2005; Gotoh et al. 2007; Miyauchi et al. 2009). In addition, activation of GPR120, which is expressed endogenously in murine enteroendocrine (STC-1) cells, promotes glucagon-like peptide-1 (GLP-1) and cholecystokinin secretion (Tanaka et al. 2008a, b). GLP-1 secretion via GPR120 signaling increases insulin secretion in vivo (Hirasawa et al. 2005). Furthermore, activation of GPR120 inhibited serum deprivation-induced apoptosis in STC-1 cells (Katsuma et al. 2005). Therefore, research into GPR40 and GPR120 as potential drug targets for diabetes has received considerable attention (Suzuki et al. 2008; Swaminath 2008), and the development of selective ligands for these receptors would be valuable tools with which to investigate the pharmacological and physiological functions of fatty acid receptors.

Although a number of GPR40 ligands have been identified and evaluated (Briscoe et al. 2003; Tikhonova et al. 2007; Bharate et al. 2008; Davi and Lebel 2008; Hara et al. 2009; Hirasawa et al. 2008a; Suzuki et al. 2008), these compounds have activated not only GPR40 but also GPR120. Moreover, there are a few reports on GPR120 synthetic ligands that were identified in our study (Suzuki et al. 2008). Hence, the pharmacology of GPR40 and GPR120 is not yet fully understood because of lack of selective ligands for these receptors. In order to discover selective pharmacological probes for these receptors, more than 80 natural compounds were screened, together with a reference compound, with the goal of finding selective, potent agonists or antagonists by monitoring intracellular signaling using inducible and stable cell lines expressing GPR40 and GPR120, respectively.

In this study, we identify and characterize a selective agonist for GPR40 as well as selective partial agonists for GPR120. Furthermore, we demonstrate that these compounds can be useful pharmacological probes to investigate the physiological functions of GPR120 by measuring GLP-1 secretion from STC-1 cells, which expresses GPR120 endogenously.

Materials and methods

Compounds

Plant materials Fruiting bodies of *Albatrellus ovinu* were collected in Musashimurayama, Japan, in October 2000 and identified by Mr. Yasuhiko Gotoh. A voucher specimen was deposited at the Institute of Food Culture, Kurashiki Sakuyo University. Fruit bodies of *Albatrellus dispansus* were collected in October 2002 in Okutama, Tokyo, Japan, and identified by Mr. Yasuhiko Gotoh. A voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University. Fruiting bodies of *Albatrellus confluens* were collected in October 2004 in Nakatsugawa, Gifu, Japan, and identified by Mrs. Makiko Nukata. A voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University. Grifolin, neogrifolin, and 3-hydroxyneogrifolin were isolated from *A. ovinu* according to a previously reported procedure (Nukata et al. 2002). Grifolic acid, grifolic acid methyl ether, and grifolin monomethyl ether were isolated from *A. dispansus* according to a previously reported procedure (Hashimoto et al. 2005; Ishii et al. 1988). Grifolin dimethyl ether was synthesized from grifolin according to a previously reported procedure (Vrkoc et al. 1997). These compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and stored at −20°C. The structures of these compounds are shown in Table 1.

Chemicals MEDICA16 was purchased from Sigma (St. Louis, MO, USA). All other materials were from standard sources and of highest purity that is available commercially.

Plasmids

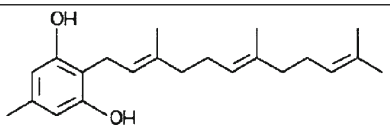
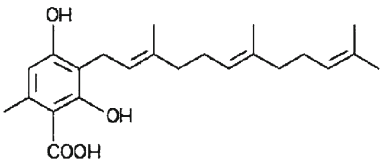
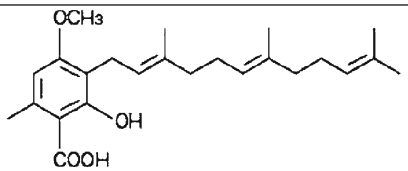
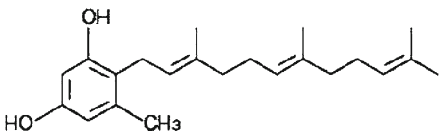
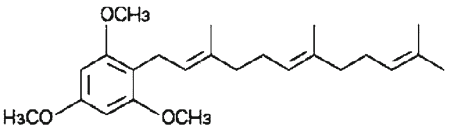
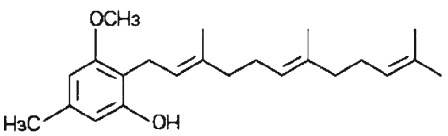
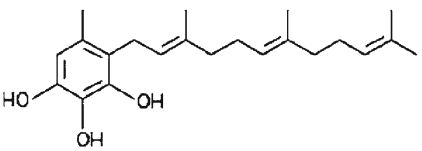
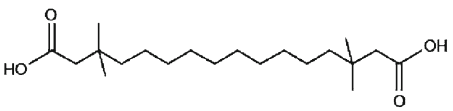
The FLAG-human GPR40 (hGPR40)/pcDNA5/FRT/TO plasmid was prepared as described previously (Hirasawa et al. 2005). Briefly, hGPR40 complementary DNA (cDNA) was obtained by polymerase chain reaction (PCR) using genomic DNA as a template and ligated into the multicloning site of the mammalian expression vector pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA, USA) together with an N-terminal FLAG-tag. To construct the human GPR120 (hGPR120)-$G\alpha_{16}$/

pcDNA5/FRT plasmid, hGPR120 cDNA and $G\alpha_{16}$ were obtained by PCR using genomic DNA as a template, and $G\alpha_{16}$ cDNA was amplified and ligated into the multicloning site of the mammalian expression vector pcDNA5/FRT (Invitrogen). hGPR120 cDNA was then ligated into the multicloning site of $G\alpha_{16}$/pcDNA5/FRT.

Cell lines

Flp-In™ T-REx™-293 (T-REx 293) cells and Flp-In™-293 (Flp-in 293) cells (Invitrogen) were used to develop inducible and stable cell lines T-REx GPR40 and Flp-in GPR120, respectively. T-REx 293 cells were transfected with FLAG-GPR40/pcDNA5/FRT/TO using Lipofectamine™ Reagent (Invitrogen) and selected with Dulbecco's modified Eagle's medium (DMEM) (Sigma), which had been supplemented with 10% fetal bovine serum (FBS), 10 μg/ml blasticidin S (Funakoshi, Tokyo, Japan), and 100 μg/ml hygromycin B (Gibco BRL, Grand Island, NY, USA). GPR40 protein expression was induced by adding 10 μg/ml of doxycycline hydrate (Dox) (Sigma) for over 24 h. T-REx 293 cells were

Table 1 The chemical structure of compounds investigated in this study

Compounds	Structure
Grifolin	
Grifolic acid	
Grifolic acid methyl ether	
Neogrifolin	
Grifolin dimethyl ether	
Grifolin monomethyl ether	
3-Hydroxyneogrifolin	
MEDICA16	

routinely cultured in DMEM supplemented with 10% FBS, 100 μg/ml zeocin (Invitrogen), and 10 μg/ml blasticidin S. Flp-in 293 cells were routinely cultured in DMEM supplemented with 10% FBS and 100 μg/ml zeocin. Flp-in 293 cells were transfected with hGPR120-Gα_{16}/pcDNA5/FRT using Lipofectamine™ Reagent and selected with DMEM that had been supplemented with 10% FBS and 100 μg/ml zeocin. STC-1 cells were cultured in DMEM containing 15% horse serum and 2.5% FBS. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Intracellular $[Ca^{2+}]_i$ measurement

Cells were seeded at a density of 2×10^5 cells/well on collagen-coated 96-well plates, incubated at 37°C for 21 h, and then incubated in Hanks' balanced salt solution (HBSS, pH 7.4) containing Calcium Assay Kit Component A (Molecular Devices, Sunnyvale, CA, USA) for 1 h at room temperature. Compounds used in the fluorometric imaging plate reader (FLIPR, Molecular Devices) assay were dissolved in HBSS (1% DMSO) and prepared in another set of 96-well plates. These plates were set on the FLIPR, and mobilization of $[Ca^{2+}]_i$ evoked by agonists was monitored. Antagonists were added 10 min before the addition of α-LA. Data analysis was performed using Igor Pro (WaveMetrics, Lake Oswego, OR, USA).

The pA_2 values for the partial agonists were calculated as described by Arunlakshana and Schild (1959). Briefly, the concentration of agonist are denoted $[A_0]$ in the absence of antagonist and $[A_X]$ in the presence of concentration of antagonist B $[B_X]$. Applying the law of mass action leads to

$$[B_X]/K_B = ([A_X]/[A_0]) - 1 \quad (1)$$

where $[A_X]/[A_0]$ is the agonist dose ratio for each concentration $[B_X]$, and K_B is the dissociation constant of the antagonist. Equation 1 is equivalent to

$$\log([A_X]/[A_0] - 1) = \log[B_X] - \log K_B \quad (2)$$

Under equilibrium conditions, the pA_2 is equal to $-\log K_B$.

ERK phosphorylation

ERK phosphorylation induced by the indicated compounds was measured as described previously (Hirasawa et al. 2005). Briefly, cells were serum-starved for 2 h and treated

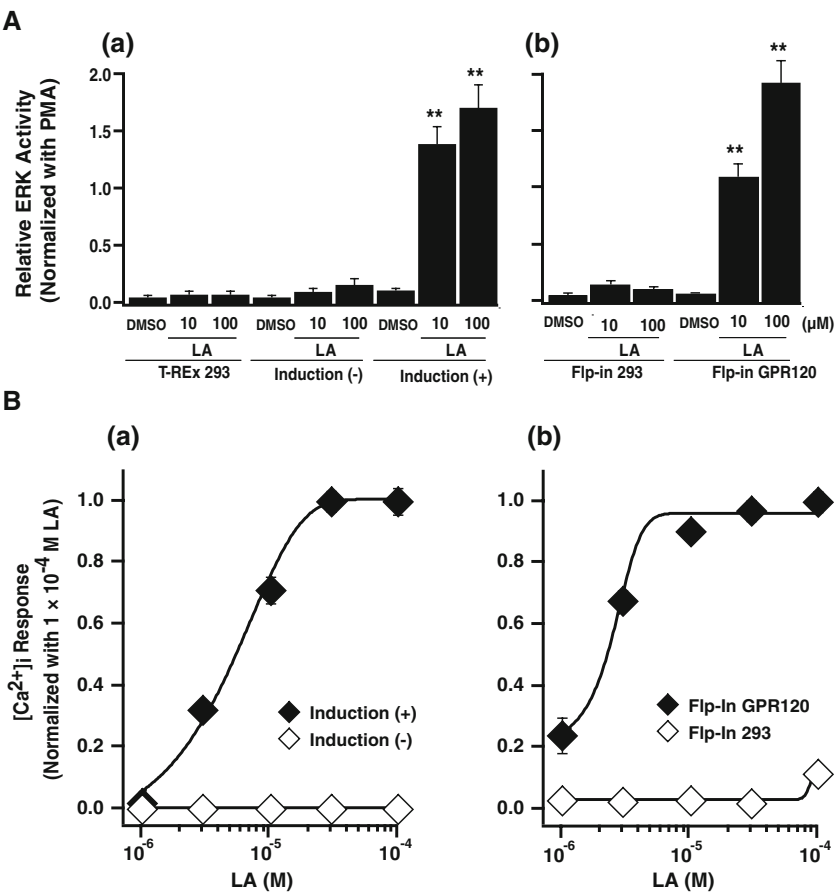


Fig. 1 Effects of LA on GPR40 or GPR120. **a** Effects of LA on ERK activation in T-REx GPR40 cells and Flp-in GPR120 cells. T-REx GPR40 cells that had been incubated with Dox [induction (+)] or without Dox [induction (−)] and T-REx 293 cells *(a)* and Flp-in GPR120 cells or Flp-in 293 cells *(b)* were stimulated with LA at 10 or 100 μM final concentration. Cell lysates were analyzed by Western blotting using anti-phospho- and anti-total-kinase antibodies. Data are means±SE of three independent experiments. $^{**}P<0.01$ vs. DMSO. **b** Effects of LA on $[Ca^{2+}]_i$ mobilization in T-REx GPR40 cells and Flp-in GPR120 cells. T-REx GPR40 cells that had been incubated with Dox [induction (+)] or without Dox [induction (−)] and T-REx 293 cells *(a)* and Flp-in GPR120 cells or Flp-in 293 cells *(b)* were stimulated with various concentrations of LA

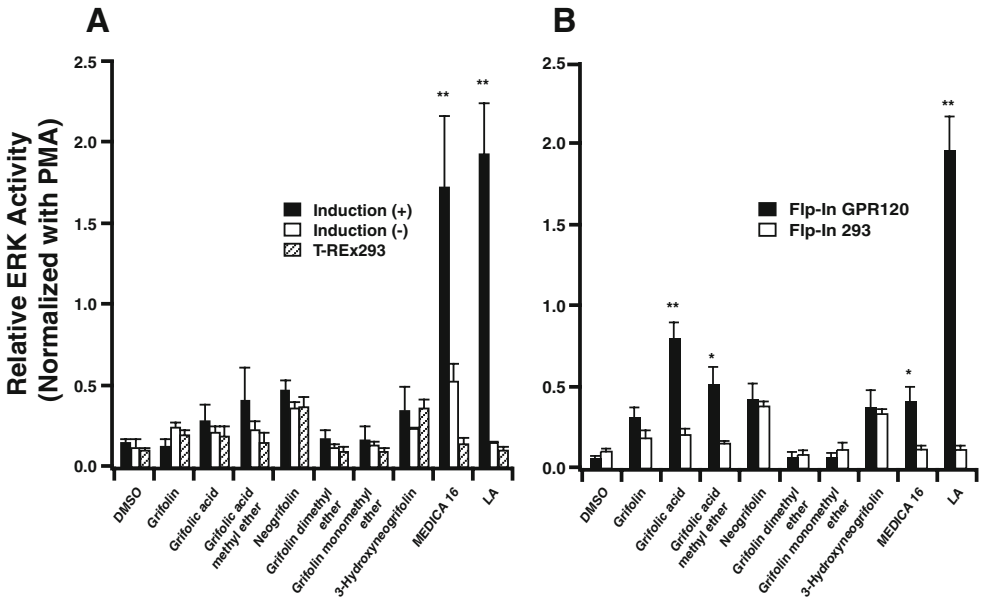


Fig. 2 Effects of each compound on ERK activation in T-REx GPR40 and Flp-in GPR120 cells. T-REx GPR40 cells that had been incubated with Dox [induction (+)] or without Dox [induction (−)] and T-REx 293 cells (**a**) and Flp-in GPR120 cells or Flp-in 293 cells (**b**) were stimulated with each compound at 100 μM final concentration. Cell lysates were analyzed by Western blotting using anti-phospho- and anti-total-kinase antibodies. Data are means±SE of three independent experiments. *$P<0.05$ and **$P<0.01$ vs. DMSO

with each compound that was being tested at a concentration of 100 μM. After 10 min of incubation with each compound, total cell extracts were prepared and subjected to Western blotting using anti-phospho- and anti-total-kinase antibodies.

GLP-1 secretion

GLP-1 secretion from STC-1 cells was measured as described previously (Hirasawa et al. 2005). Briefly, STC-1 cells were seeded at a density of 4×10^5 cells/well on 24-well plates, incubated for 48 h at 37°C in the medium, and stimulated with compounds in HBSS for 1 h at 37°C. After incubation, supernatants were collected, and the concentration of GLP-1 was determined by enzyme immunoassay with Rat GLP-1 ELISA Kit Wako (Wako, Osaka, Japan).

Statistical analysis

The level of significance for the difference between sets of data was assessed using an unpaired Student's *t* test. Data were expressed as means±SE. $p<0.05$ was considered as statistically significant.

Results

Analysis of cell lines expressing GPR40 and GPR120

In order to identify novel ligands for GPR40 and GPR120, a Dox-inducible cell line expressing hGPR40 (T-REx GPR40) and a stable cell line expressing hGPR120 (Flp-in GPR120) were developed as described in "Materials and

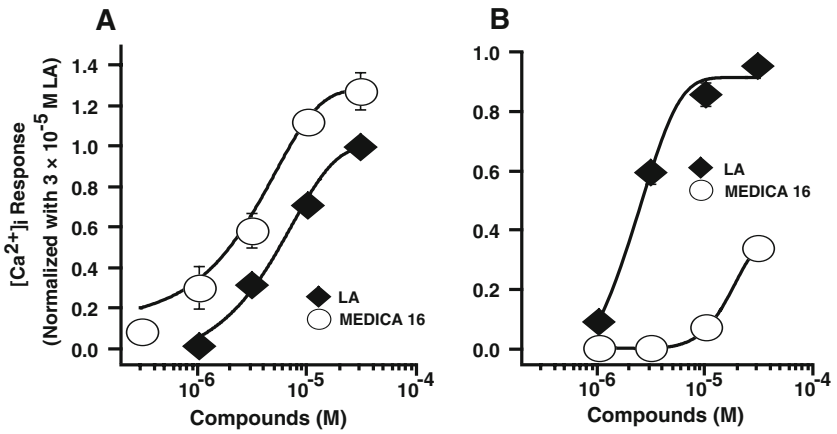


Fig. 3 Effects of MEDICA16 on $[Ca^{2+}]_i$ mobilization in T-REx GPR40 and Flp-in GPR120 cells. T-REx GPR40 cells that had been incubated with Dox [induction (+)] (**a**) and Flp-in GPR120 cells (**b**) were stimulated with various concentrations of LA or MEDICA16

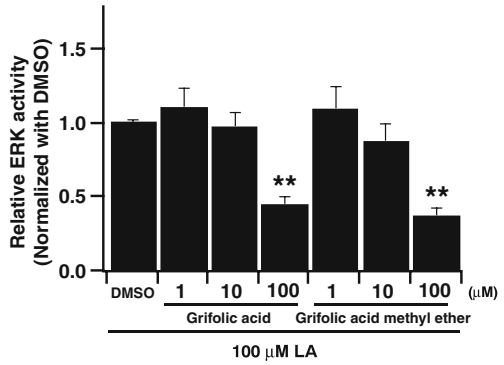


Fig. 4 Inhibition effects of test compounds on ERK activation in Flp-in GPR120 cells. Flp-in GPR120 cells were stimulated with LA at 100 μM final concentration. After stimulation with LA, cells were loaded for 5 min with test compounds at the indicated concentrations. Data are means±SE of three independent experiments. $^{**}P<0.01$ vs. DMSO

methods". To determine whether these two receptors possess the agonist responsiveness, ERK and $[Ca^{2+}]_i$ responses were measured after stimulation of these cell lines with LA, which is known to activate GPR40 and GPR120 (Briscoe et al. 2003; Itoh et al. 2003; Hirasawa et al. 2005). LA increased ERK and $[Ca^{2+}]_i$ responses in a dose-dependent manner in T-REx GPR40 cells incubated with Dox [Induction (+); Fig. 1a, (a) and b, (a)] and in Flp-in GPR120 cells [Fig. 1a, (b) and b, (b)]; however, T-REx 293 cells, T-REx GPR40 cells incubated without Dox [induction (−)], and Flp-in 293 cells showed only a basal level of activation after addition of LA. These results indicated that the activation of ERK and $[Ca^{2+}]_i$ responses induced by LA in these cell lines was mediated by these receptors and was not a nonspecific effect.

Screening for the GPR40 and GRP120 ligands

Using the cell lines described herein, in order to identify selective ligands for GPR40 and GPR120, 80 natural compounds were screened, together with the reference compound MEDICA16, which is known to activate GPR40, by monitoring ERK activity in T-REx GPR40 cells and in Flp-in GPR120 cells. Among the 80 natural compounds, a series of grifolin derivatives isolated from plant materials (Table 1) was identified, which possess the ability to activate GPR120. As shown in Fig. 2a, none of the grifolin derivatives tested activated ERK in T-REx GPR40 cells incubated with Dox. As shown in Fig. 2b, the grifolin derivatives, grifolic acid and grifolic acid methyl ether, activated ERK in Flp-in GPR120 cells, although the potencies of these two compounds were much lower than that of LA. MEDICA16 increased the ERK activity both in T-REx GPR40 cells incubated with Dox and in Flp-in GPR120 cells; however, the effect of this compound on ERK activity was more potent in the T-REx GPR40 cells (Fig. 2a) compared to the Flp-in GPR120 cells (Fig. 2b). Thus, the screens identified specific ligands for GPR40 and GPR120.

Pharmacological effects of the ligands on GPR40 and GPR120

The effects of MEDICA16, grifolic acid, and grifolic acid methyl ether on activation of GPR40 and GPR120 were next examined in greater detail. In accordance with the result of the ERK assay, MEDICA16 potently increased $[Ca^{2+}]_i$ in a dose-dependent manner in T-REx GPR40 cells incubated with Dox, compared to the response in Flp-in GPR120 cells (Fig. 3a, b), indicating that MEDICA16 could be a selective agonist for GPR40.

As shown in Fig. 2b, some grifolin derivatives activated GPR120, but their potency was far less than that of LA. Thus, we speculated that they might act as partial agonists for GPR120, so we next examined whether grifolic acid and grifolic acid methyl ether could act as antagonists for

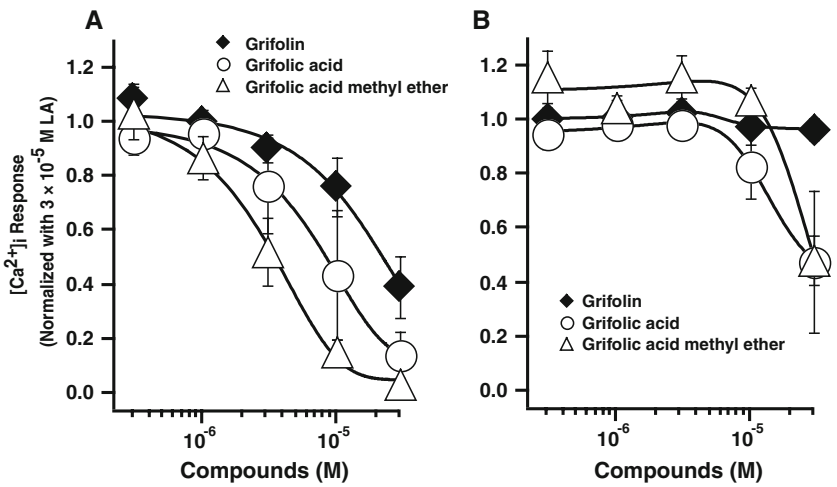


Fig. 5 Inhibition curves of $[Ca^{2+}]_i$ response induced by LA with test compounds in Flp-in GPR120 or T-REx GPR40 cells. Flp-in GPR120 (**a**) and T-REx GPR40 cells (**b**) were stimulated with LA at 100 μM final concentration. After stimulation with LA, test compounds were loaded at the indicated concentrations. Data are means ±SE of three independent experiments

Table 2 Comparison of apparent pA_2 and relative affinity for grifolic acid and grifolic methyl ether

Antagonist	Number of experiments	Apparent pA_2 ($\times 10^{-6}$M)	Relative affinity
Grifolic acid	3	4.95±0.1	1.00[a]
Grifolic acid methyl ether	3	5.01±0.1	0.79 NS

Apparent K_B values were converted to apparent pA_2 values as described in "Materials and methods". Results are means±SE of three independent experiments

NS not significant

[a] Grifolic acid, arbitrary set at 1

GPR120. As shown in Fig. 4, these two compounds inhibited LA-induced ERK phosphorylation in a dose-dependent manner in Flp-in GPR120 cells. In addition, these compounds together with grifolin inhibited LA-induced $[Ca^{2+}]_i$ in a dose-dependent manner. On the other hand, grifolic acid and grifolic acid methyl ether inhibited LA-induced $[Ca^{2+}]_i$ in T-REx GPR40 cells incubated with Dox, but their inhibitory effects were less than that seen in Flp-in GPR120 cells (Fig. 5a, b). In addition, the pA_2 values of these two compounds for GPR120 were calculated as described in "Materials and methods". The relative affinity of grifolic acid methyl ether and that of grifolic acid was compared by dividing the apparent K_B of grifolic acid methyl ether by that of grifolic acid (Table 2). This result revealed that the antagonistic effects of these two compounds were not significantly different. Other grifolin derivatives, specifically neogrifolin, grifolin dimethyl ether, grifolin monomethyl ether, and 3-hydroxyneogrifolin, did not inhibit ERK or $[Ca^{2+}]_i$ responses in the two cell lines (data not shown). These results indicated that some grifolin derivatives could be selective partial agonists for GPR120.

The biological effects of grifolic acid and grifolic acid methyl ether on GPR120 were next examined by measuring GLP-1 secretion from STC-1 cells. Stimulation of GPR120 has been shown to promote secretion of GLP-1 from STC-1 cells (Hirasawa et al. 2005). Therefore, the effects of these compounds on GLP-1 secretion from STC-1 cells were examined. As shown in Fig. 6, LA, grifolic acid, and grifolic acid methyl ether stimulated GLP-1 secretion in a dose-dependent manner. On the other hand, MEDICA16 stimulation did not increase GLP-1 secretion significantly. Thus, the effects of these compounds on GPR120 activation in Flp-in GPR120 cells correlated well with their ability to induce GLP-1 secretion from STC-1 cells via GPR120.

Discussion

This report identifies and characterizes selective ligands for GPR40 and GPR120 that were isolated from natural compounds. The compounds, together with the reference compound MEDICA16, were identified by measuring intracellular signals ($[Ca^{2+}]_i$ and ERK responses) in inducible and stable expression cell lines. These cell lines were established previously, and the expression and function of these receptors were examined (Hirasawa et al. 2005, 2008b; Hara et al. 2009), leading to the development of assays that could discriminate whether signals induced by various ligands were mediated by specific receptors. This led to identification of the selective GPR40 ligand MEDICA16 and the selective GPR120 ligands grifolic acid and grifolic acid methyl ether.

MEDICA16 is known to activate GPR40 (Kotarsky et al. 2003), and it is an experimental anti-obesity compound (Bar-Tana et al. 1985). However, the selectivity of MEDICA16 between GPR40 and GPR120 has not yet been elucidated in the literature. In this study, the effect of MEDICA16 on GPR40 activation was found to be more potent than its effect on GPR120. Hence, MEDICA16 may act as a selective agonist that could be used as a selective pharmacological probe for GPR40. Moreover, the results of screening 80 natural compounds demonstrated that two grifolin derivatives, grifolic acid and grifolic acid methyl

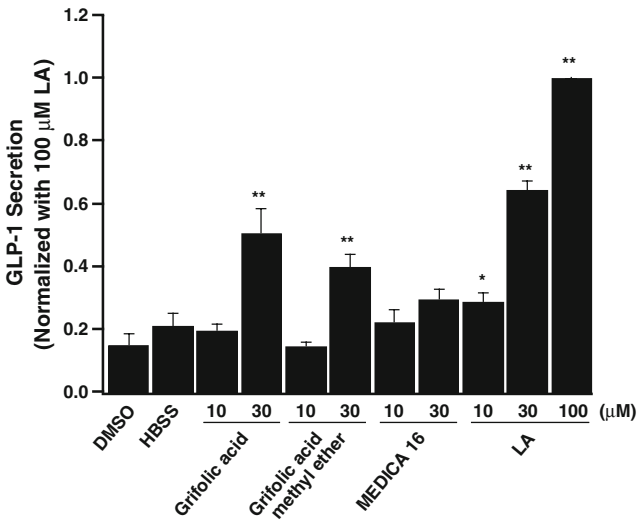


Fig. 6 Effects of test compounds on GLP-1 secretion in STC-1 cells. STC-1 cells were incubated with the test compounds for 60 min at 37° C in HBSS (30 μM). Data are means±SE of three or four independent experiments. *P<0.05 and **P<0.01 vs. DMSO

ether, which were isolated from the fresh fruiting bodies of the mushroom *A. confluens* or were synthesized with chemical modification of these compounds, possessed pharmacological activity by acting through GPR120. This study confirms that grifolic acid and grifolic acid methyl ether not only activated ERK and $[Ca^{2+}]_i$ responses through GPR120 signaling, but they also inhibited responses induced by LA in GPR120-expressing cells. Therefore, these two compounds may act as novel partial agonists for GPR120. In addition, they may also act as antagonists for GPR40, but their potencies as antagonists were much less pronounced. These two compounds showed both agonistic and antagonistic activities when used to stimulate GPR120-expressing cells. However, additional grifolin derivatives (neogrifolin, grifolin dimethyl ether, grifolin monomethyl ether, and 3-hydroxyneogrifolin) did not induce any responses in either receptor. In accordance with previous reports (Itoh et al. 2003; Hirasawa et al. 2005), these results also indicated that the carboxyl group of grifolin derivatives was indispensable for the activation of GPR120. In addition, although further investigations of GPR120 ligand selectivity for GPR120 are needed, these results provide useful information on structure–activity relationships between GPR120 and its ligands.

The biological properties of these compounds on GLP-1 secretion via GPR120 were further investigated using STC-1 cells, which express GPR120 endogenously. Their effects on GLP-1 cells correlated well with their effect on $[Ca^{2+}]_i$ and ERK responses in Flp-in GPR120 cells. In contrast, MEDICA16, which selectively activated ERK and $[Ca^{2+}]_i$ response in T-REx GPR40 cells, did not induce GLP-1 secretion from STC-1 cells. These results indicated that grifolic acid and grifolic acid methyl ether could be useful probes with which to discriminate between the pharmacological effects of GPR40 and GPR120.

Grifolin derivatives have been shown to possess a broad spectrum of biological effects, such as inhibiting growth and inducting significant apoptosis in some cancer cell lines (Ishii et al. 1988; Zechlin et al. 1981; Sugiyama et al. 1992). They also showed hypocholesterolemic action in rats fed a high-cholesterol diet (Sugiyama et al. 1992). However, the relationship between GPR120 and grifolin derivatives has not been reported. Hence, the effect of these compounds on GLP-1 secretion via GPR120 represents a novel biological property of grifolin derivatives.

Recently, the synthetic 4-(benzylamino)dihydrocinnamic acid derivative (GW9508) and the 4-phenethynyldihydrocinnamic acid derivative (TUG-424) were shown to possess agonistic activity towards GPR40 (Briscoe et al. 2006; Christiansen et al. 2008). GW9508 showed agonistic activity towards GPR120 and GPR40. On the other hand, only the effect on GPR40 has been examined for TUG-424. In addition, 4-{4-[2-(phenyl-2-pyridinylamino)ethoxy]phenyl}butyric acid (compound 12) have been identified as selective ligands for GPR120, but their selectivity between GPR40 and GPR120 was evaluated only using an overexpression system (Suzuki et al. 2008). Thus, the selectivity of various ligands between GPR40 and GPR120 remains to be fully elucidated. At present, the pharmacology of FFARs, including GPR40 and GPR120, is not fully understood despite intensive research efforts, mainly because there are few specific and selective ligands for GPR120. Thus, the compounds identified in this study could be used as novel selective probes capable of distinguishing pharmacological and physiological effects related to signaling through GPR40 and GPR120.

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