Acta Pharmacologica Sinica (2015) 36: 813–820
www.nature.com/aps

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Original Article

Omega-3 fatty acids induce Ca^{2+} mobilization responses in human colon epithelial cell lines endogenously expressing FFA4

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Aim: Free fatty acid receptor 4 (FFA4; formerly known as GPR120) is the G protein-coupled receptor (GPCR) for omega-3 polyunsaturated fatty acids. FFA4 has been found to express in the small intestines and colons of mice and humans. In this study we investigate the effects of omega-3 polyunsaturated fatty acids on FFA4 in human colon epithelial cells *in vitro*.
Methods: HCT116 and HT-29 human colon epithelial cell lines endogenously expressing FFA4 were used. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in fura 2-AM-loaded cells with fluorescence spectrophotometry. RT-PCR and immunohistochemistry were used to detect FFA4.
Results: Ten to 100 μmol/L of omega-3 polyunsaturated fatty acids α-linolenic acid (αLA) or eicosapentaenoic acid (EPA) induced dose-dependent $[Ca^{2+}]_i$ increase in HCT116 and HT-29 cells, whereas docosahexaenoic acid (DHA) had no effect. In addition, the omega-6 fatty acids linoleic acid and γ-linoleic acid also dose-dependently increase $[Ca^{2+}]_i$, but the mono-unsaturated fatty acid oleic acid and saturated fatty acids such as stearic acid and palmitic acid had no effect. In HCT116 and HT-29 cells, the αLA-induced $[Ca^{2+}]_i$ increase was partially inhibited by pretreatment with EGTA, phospholipase C inhibitor edelfosine, cADPR inhibitors 8-bro-cADPR or DAB, and abolished by pretreatment with Ca^{2+} ATPase inhibitor thapsigargin, but was not affected by $G_{i/o}$ protein inhibitor PTX or IP_3R inhibitor 2-APB.
Conclusion: Omega-3 and omega-6 long-chain polyunsaturated fatty acids (C18-20) induce Ca^{2+} mobilization responses in human colonic epithelial cells *in vitro* through activation of FFA4 and PTX-insensitive $G_{i/o}$ protein, followed by Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} influx across the plasma membrane.

Keywords: long-chain fatty acid; omega-3; α-linolenic acid; FFA4; Ca^{2+} mobilization; cADPR; human colon epithelial cells

Acta Pharmacologica Sinica (2015) 36: 813–820; doi: 10.1038/aps.2015.29; published online 25 May 2015

Introduction

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are essential fatty acids with a double bond between the third and fourth carbon atoms from the methyl end (ω end) of the fatty acid carbon chain[1]. Because these fatty acids cannot be synthesized by the human body, the dietary intake of ω-3 PUFAs such as α-linolenic acid (ALA, C18:3), eicosapentaenoic acid (EPA, C20:5), and docosahexaenoic acid (DHA, C22:6) is important. ω-3 PUFAs were initially shown to be beneficial in epidemiological studies conducted in Greenland Inuits[2]. They are clinically prescribed for the prevention of myocardial infarction and the treatment of hypertriglyceridemia[3, 4]. Omega-3 PUFAs are also popularly consumed as functional foods; however, the pharmacological effects of ω-3 PUFAs differ, especially with respect to their anti- and pro-inflammatory effects on the colon[5–9]. These different effects of ω-3 PUFAs cannot be explained by the accepted mode of action of ω-3 PUFAs, that is, by the biochemical competition of ω-3 PUFAs on enzymes required for inflammatory mediator biosynthesis[10] and the activation of anti-inflammatory PPARγ[1, 11].

Recently, free fatty acid receptor 4 (FFA4; formerly known as GPR120) was found to act as a G protein-coupled receptor (GPCR) for ω-3 PUFAs[12–16]. FFA4 was originally cloned as an orphan GPCR[17], but in 2005, long-chain fatty acids were observed to induce increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and FFA4 internalization in HEK293 cells exogenously expressing

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Received 2015-02-02 Accepted 2015-03-05

FFA4[13]. FFA4 responds to saturated free fatty acids (C14 to C18) and unsaturated free fatty acids (C16 to C22), including αLA, EPA, and DHA, in HEK293 cells, although αLA was the best agonist in terms of Ca^{2+} response[13]. Furthermore, FFA4 expression is abundant in small and large intestines, lungs, and adipose tissues and on macrophages[12, 13, 15, 18]. In humans, there are two alternative splicing variants of FFA4, short- and long-form FFA4[19, 20]; the long form has additional 16 amino acids in the third intracellular loop compared with the short-form FFA4[19, 21]. In one study, a similar Ca^{2+} mobilization response to linoleic acid (C18:2n-6) was observed for both isoforms; however, the saturated medium-chain fatty acid capric acid (C10:0) only showed partial agonism for short-form FFA4[22]. In another study, only long-form FFA4 induced β-arrestin-mediated responses, whereas short-form FFA4 induced G protein-mediated Ca^{2+} signaling and β-arrestin-mediated FFA4 phosphorylation and internalization [21]. Interestingly, in the gastrointestinal tract, only the colon expressed both forms of FFA4[22].

Since the identification of FFA4 as a GPCR for long-chain PUFAs, FFA4 has been reported to be involved in the fatty acid-stimulated release of glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) from enteroendocrine cells[13, 23], and ω-3 PUFA-induced anti-inflammatory responses in macrophages[15]. Furthermore, ω-3 PUFA-glucose uptake and adipogenesis in adipocytes have been investigated in obesity and type II diabetes[14, 15]. Additionally, genetic studies have shown that FFA4 knockout mice and humans possessing a FFA4 polymorphism associated with reduced signaling are prone to obesity[14]. Because FFA4 agonists might constitute unique and novel treatments for diabetes by improving insulin sensitivity and reducing metabolic inflammation, a number of selective FFA4 agonists have been developed[24–30]. However, the functions of FFA4 in the colon have not been investigated.

Because a variety of fatty acids are released during digestion in the gastrointestinal tract, the specificity of endogenous FFA4 for natural nutrient fatty acids in the small and large intestines must be investigated. The different effects of ω-3 PUFAs on colonic inflammation suggested that the different response in colon might be caused by different responses of endogenous FFA4 to individual ω-3 PUFAs in the colon. Therefore, in the present study, we examined the effects of several ω-3 PUFAs in HCT116 and HT-29 human colon epithelial cell lines as a model system of colonic epithelial cells that endogenously express FFA4.

Materials and methods

Materials

αLA, DHA, capric acid, palmitic acid, oleic acid, Fura 2-AM, EGTA, 2-aminoethoxydiphenylborane (2-APB), pertussis toxin (PTX), 8-bromo-cyclic adenosine diphosphate ribose (8-Bro-cADPR), 2,2′-dihydroxyazobenzene (DAB), and thapsigargin were purchased from Sigma-Aldrich (St Louis, MO, USA). EPA, lauric acid, myristic acid, stearic acid, linoleic acid, γ-linolenic acid, TUG-891, and edelfosine were obtained from Cayman (Ann Arbor, MI, USA).

Cell culture

HCT116 and HT-29 human colon cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in high-glucose DMEM containing 10% (*v*/*v*) fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate at 37 °C in a humidified 5% CO_2/95% air incubator[31].

Measurement of intracellular Ca^{2+} concentrations

Cells were trypsin-digested, allowed to sediment, and resuspended in HEPES-buffered medium consisting of 20 mmol/L HEPES (pH 7.4), 103 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 0.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 15 mmol/L glucose. The cells were then incubated for 40 min with 5 μmol/L Fura 2-AM. $[Ca^{2+}]_i$ was estimated by measuring the changes in Fura 2 fluorescence at an emission wavelength of 510 nm and excitation wavelengths of 340 nm and 380 nm every 0.1 s using a F4500 fluorescence spectrophotometer (Hitachi, Japan). The ratios of fluorescence intensities (λ_{340}/ λ_{380}) at these two wavelengths were used as an indicator of $[Ca^{2+}]_i$, as previously described[32].

Reverse transcription-PCR (RT-PCR)

To assess the expression of FFA4 in HCT116 and HT-29 cells by RT-PCR, first-strand complementary DNA (cDNA) was synthesized using total RNA isolated with TRIzol reagent (Invitrogen, USA). cDNA synthesis from total RNA (1 μg) was performed using Oligo(dT) 15 primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Synthesized cDNA products and primers for free fatty acid receptor 1 (FFA1) and FFA4 were used for PCR using an Eppendorf Mastercycler gradient PCR machine (Hamburg, Germany). The primers used to amplify 210, 249, 402, 303, and 238 bp fragments of FFA1, FFA4, short-form FFA4, long-form FFA4, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: FFA1 (sense 5′-GTG TCA CCT GGG TCT GGT CT-3′, antisense 5′-GAG CAG GAG AGA GAG GCT GA-3′), FFA4 (sense 5′-CTT CTT CTC CGA CGT CAA GG-3′, antisense 5′-AGA GGG AGA GCG CTG ATG AA-3′), short-form FFA4 (sense 5′-CGA TTT GCA CAC TGA TTT GGC-3′, antisense 5′-TGC ACA GTG TCA TGT TGT AGA-3′), long-form FFA4 (sense 5′-GCT GTC GTG ACT CAC AGT G-3′, antisense 5′-TGC ACA GTG TCA TGT TGT AGA-3′) and GAPDH (sense 5′-GAG TCA ACG GAT TTG GTC GT-3′, antisense 5′-TTG ATT TTG GAG GGA TCT CG-3′). The PCR reactions were performed with denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and 27 cycles for FFA1, annealing at 55 °C for 30 s and 30 cycles for FFA4, annealing at 60 °C for 30 s and 25 cycles for short-form FFA4, long-form FFA4, and GAPDH, and elongation at 72 °C for 30 s. Aliquots of the PCR products (7 μL) were electrophoresed in 1.2% agarose gels and stained with ethidium bromide[33].

Immunocytochemistry

Cells were labeled with antibodies against FFA4 (NBP1-00858,

1:500, Novus Biologicals, LLC, CO, USA) and β-arrestin 2 (ab54790, 1:500, Abcam, Cambridge, MA, USA). The secondary antibody used was Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000, Jackson ImmunoResearch, West Grove, PA, USA) and Rhodamine Red™-X-conjugated anti-mouse IgG (1:1000, Jackson ImmunoResearch, West Grove, PA, USA). A confocal scanning module (LSM510, Carl Zeiss, Germany) mounted on a fluorescence microscope (AXIOVERT 100 M, Carl Zeiss, Germany) and a C-Apochromat 40×/1.2 W (Carl Zeiss, Germany) was used[34].

Statistical analysis

The results are expressed as the mean±SEM for the indicated number of determinations. Significant differences were assessed using Student's *t*-test, and statistical significance was accepted for P values <0.05.

Results

FFA4 expression in human colonic epithelial cells

Two methods were used to assess FFA4 expression in HCT116 and HT-29 cells. First, RT-PCR was conducted to detect the mRNAs of FFA4 and FFA1. As shown in Figure 1A, FFA4 expression was detected in both cell lines, whereas FFA1 expression was not. Second, FFA4 protein was detected in both cell lines using a FFA4-specific antibody (Figure 1C and 1D). FFA4 protein was weakly detected in plasma membranes and cytosol. Treatment of cells with αLA, the best known agonist of FFA4, caused strong FFA4 aggregation at the cell surfaces in both cell lines (Figure 1C and 1D). Furthermore, αLA induced the co-localization of FFA4 and β-arrestin in both cell types, implying the recruitment of β-arrestin by FFA4 to cell membranes (Figure 1C and 1D). Thus, FFA4 expression and its activation by αLA were observed in both cell lines at the RNA and protein levels. Similarly, EPA and DHA induced the recruitment of β-arrestin in both cell lines (Figure 1C and 1D). However, the response to DHA was weaker than that to αLA or EPA (Figure 1C and 1D).

Due to the two splicing variants of FFA4 in the colon, specific primers for short- and long-form FFA4 were used. As shown in Figure 1B, HCT116 cells expressed only long-form FFA4, whereas HT-29 cells expressed both forms.

Intracellular Ca^{2+} increase by ω-3 PUFAs in human colonic epithelial cells

Because FFA4 couples to G proteins and increases $[Ca^{2+}]_i$ in FFA4-overexpressing cells, this Ca^{2+} response was measured in colonic epithelial cells endogenously expressing FFA4. As shown Figure 2A, the ω-3 PUFAs, αLA (C18:3) and EPA (C20:5) increased $[Ca^{2+}]_i$ in a concentration-dependent manner in HCT116 cells, whereas DHA (C22:6) did not. The ω-6 fatty, linoleic (C18:2) and γ-linoleic (C18:3) acids increased $[Ca^{2+}]_i$ (Figure 2A), whereas monounsaturated oleic acid (C18:1) and saturated fatty acids, such as stearic (C18:0), palmitic (C16:0), myristic (C14:0), and lauric (C12:0) acid, did not increase $[Ca^{2+}]_i$ in HCT116 cells (Figure 2B).

These observations suggested that there is an optimum structure for 18–20 carbon atoms with more than two double bonds and that the location of the unsaturation does not influence endogenous FFA4 activation in colonic cells. TUG-891, an FFA4-specific agonist, increased $[Ca^{2+}]_i$ in HCT116 cells (Figure 2A)[24, 26]. Although this agonist has been reported to be more potent than αLA in several cell systems[24, 26], it was less potent than other natural PUFAs in HCT116 cells (Figure 2A).

We also examined the Ca^{2+} response in HT-29 cells. As shown in Figure 2C and 2D, the ω-3 PUFAs, αLA, EPA, linoleic acid, and γ-linoleic acid increased $[Ca^{2+}]_i$ in a concentration-dependent manner, whereas DHA, stearic acid, palmitic acid, myristic acid, and lauric acid did not. Therefore, the optimum structure for the Ca^{2+} response in HT-29 cells was the same as that in HCT116 cells, that is, 18–20 carbon atoms and more than 2 double bonds. Further, the Ca^{2+} response to TUG-891 in HT-29 cells was weaker than that to αLA (Figure 2C), which was in agreement with the results for HCT116 cells. Taken together, these finding suggest that these results are typical for human colonic epithelial cells.

Effects of edelfosine, EGTA, and PTX on αLA-induced Ca^{2+} response

Because αLA was the best ligand in terms of inducing Ca^{2+} response in HCT116 and HT-29 cells and FFA4-overexpresssing systems, αLA was used in the subsequent studies. To elucidate the mechanism responsible for Ca^{2+} signaling, cells were treated with specific inhibitors or blockers of $G_{i/o}$-type G proteins, phospholipase C, and extracellular Ca^{2+}, that is, PTX, edelfosine, and EGTA, respectively.

As shown in Figure 3A, treatment of HCT116 cells with EGTA (5 mmol/L for 1 min) partially inhibited the αLA-induced $[Ca^{2+}]_i$ increases, suggesting that Ca^{2+} influx across the cell membrane partially contributes to the $[Ca^{2+}]_i$ increases. In addition, edelfosine (10 μmol/L for 6 h) also partially inhibited the αLA-induced response in HCT116 cells, suggesting the involvement of phospholipase C (Figure 3A). However, PTX (100 ng/mL for 24 h) did not inhibit the Ca^{2+} response, supporting the notion that $G_{i/o}$ proteins are not involved in the αLA-induced response in HCT116 cells (Figure 3A). These results suggest that αLA mobilizes Ca^{2+} via PTX-insensitive G proteins, likely $G_{q/11}$-type proteins, and phospholipase C and via promoting Ca^{2+} influx across the plasma membrane.

As shown in Figure 3C, treatment of HT-29 cells with edelfosine or EGTA partially inhibited the αLA-induced $[Ca^{2+}]_i$ increases, whereas treatment with PTX did not (Figure 3C). Edelfosine inhibition was stronger in HT-29 cells than HCT116 cells (Figure 3). These results suggest that αLA mobilizes Ca^{2+} via PTX-insensitive G proteins, phospholipase C, and Ca^{2+} influx across the plasma membrane in both HCT116 and HT-29 cells.

Effects of 2-APB, 8-bromo-cADPR, and thapsigargin on the αLA-induced Ca^{2+} response

Because external Ca^{2+} sequestration by EGTA blocked the Ca^{2+} response by approximately 50%, Ca^{2+} must have been released

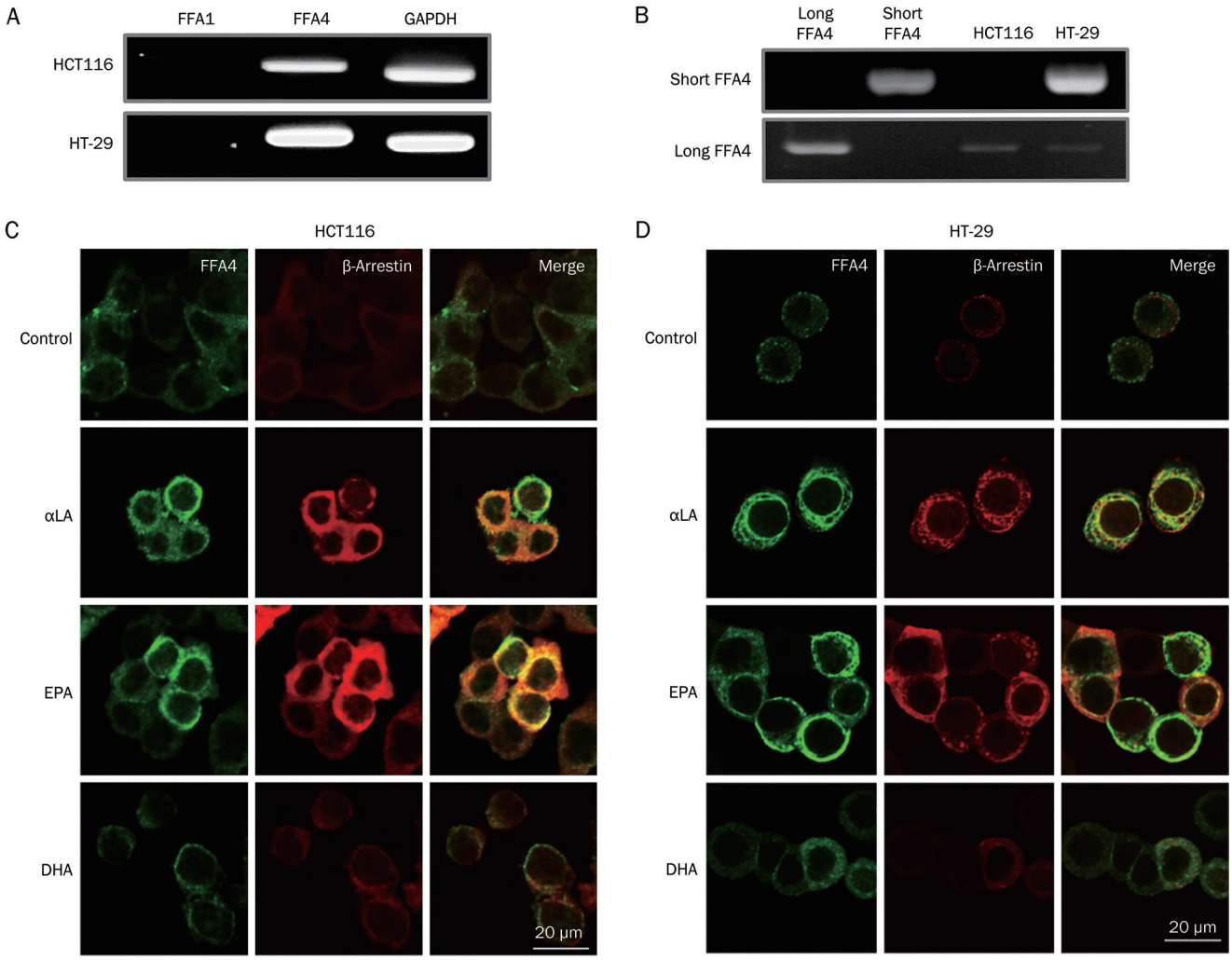

Figure 1. FFA4 expression in human colonic epithelial cells. (A) RT-PCR analyses of FFA1 and FFA4 expression in HCT116 and HT-29 human colon epithelial cells. GAPDH was used as the loading control. (B) RT-PCR analyses of long- and short-form FFA4 expression in HCT116 and HT-29 human colon epithelial cells. The long- and short-form FFA4 were used as positive controls. (C and D) Fluorescence confocal microscopy of HCT116 and HT-29 cells, respectively. FFA4 and β-arrestin 2 protein expression was detected with specific antibodies 30 min after exposing the cells to αLA, EPA, or DHA (50 μmol/L).

from internal Ca^{2+} stores, such as the endoplasmic reticulum (ER). To investigate the mechanism responsible for intracellular Ca^{2+} signaling, HCT116 and HT-29 cells were treated with specific inhibitors of inositol 1,4,5-trisphosphate receptor (IP_3R), cyclic ADP ribose receptor (cADPR), and Ca^{2+} ATPase, that is, 2-APB, 8-bro-cADPR (or DAB), and thapsigargin, respectively. 2-APB did not inhibit the αLA-induced $[Ca^{2+}]_i$ increases in HCT116 or HT-29 cells (Figure 4A, 4C). However, 8-bro-cADPR and DAB, specific inhibitors of cADPR, which is a receptor for another second messenger for Ca^{2+} signaling, partially inhibited the αLA-induced Ca^{2+} response in both HCT116 and HT-29 cells (Figure 4B, 4D). The depletion of ER Ca^{2+} stores was induced by thapsigargin, and in thapsigargin-treated cells, αLA did not induce a $[Ca^{2+}]_i$ increase in either cell line (Figure 4B, 4D), suggesting that Ca^{2+} release from the ER is a first step and necessary for Ca^{2+} influx across the plasma membrane. These results suggest that the αLA-induced $[Ca^{2+}]_i$ increases are induced by cADPR-induced Ca^{2+} release from thapsigargin-sensitive intracellular Ca^{2+} stores and Ca^{2+} influx across the plasma membrane in HCT116 and HT-29 cells.

Discussion

In the present study, the effects of long-chain fatty acids were investigated in HCT116 and HT-29 human colonic epithelial cells, which endogenously express FFA4. FFA4 expression has been observed in the small intestines and colons of mice and humans[13, 22]; the present study confirmed the expression of short- and long-form FFA4 in HT-29 cells, which is consistent with a previous report[22]. FFA4 and FFA1 can mediate rapid responses to long-chain (C12–C22) saturated and unsaturated fatty acids[30, 35, 36]. However, HCT116 and HT-29 cells do not express FFA1; thus, long-chain fatty acid-induced $[Ca^{2+}]_i$

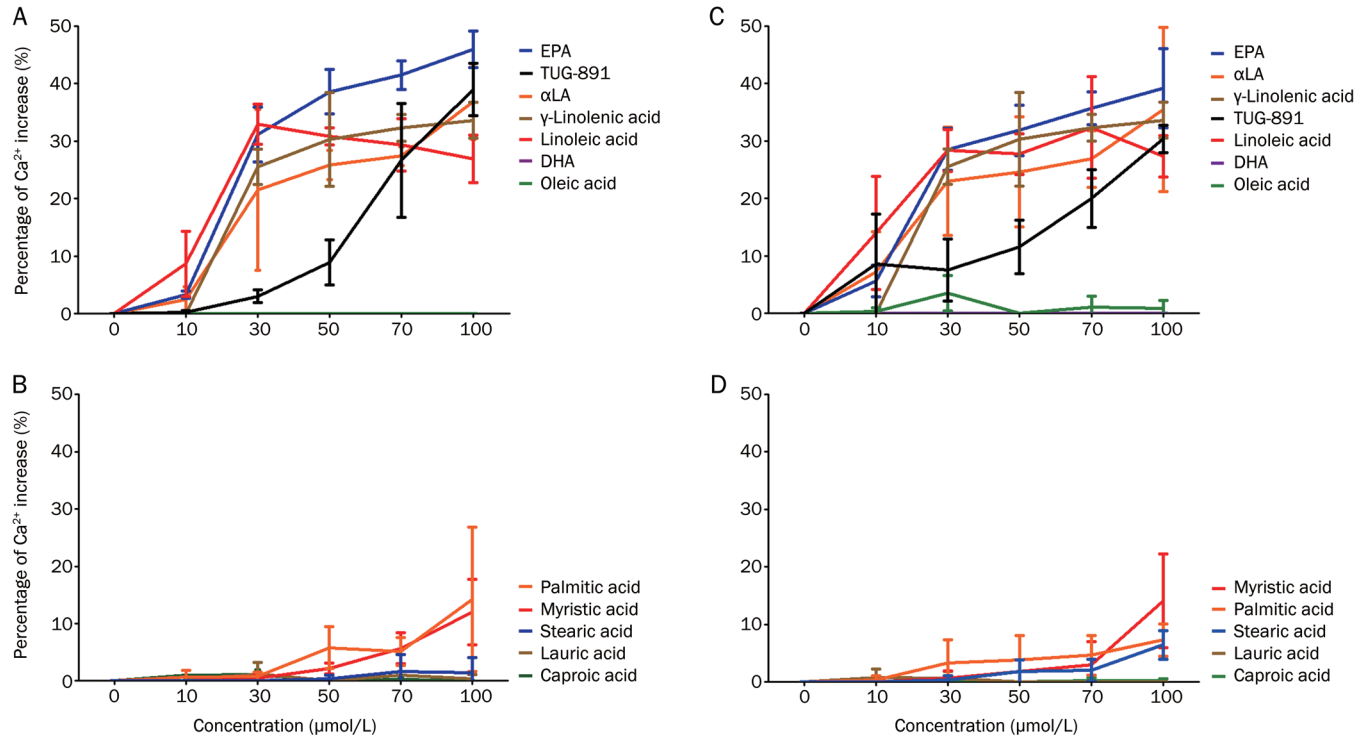

Figure 2. Concentration-response curves of different fatty acids in inducing $[Ca^{2+}]_i$ increase in HCT116 cells (A, B) and HT-29 cells (C, D). Long-chain fatty acids with 18 or more carbons were used in (A, C), whereas fatty acids with less than 18 carbons were used in (B, D). Ca^{2+} responses are shown versus the digitonin-induced maximum response.

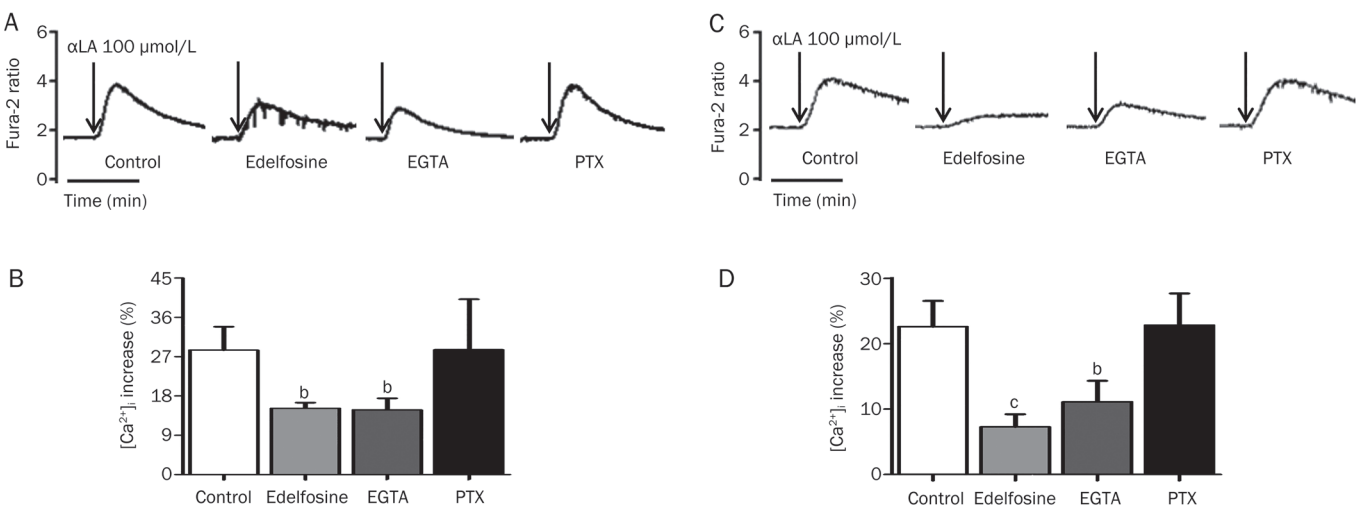

Figure 3. Effects of edelfosine, EGTA, and PTX on the αLA-induced Ca^{2+} response in human colon epithelial cells. (A and C) are the representative Ca^{2+} traces in HCT116 cells and HT-29 cells, respectively. The cells were pretreated with vehicle, edelfosine (10 μmol/L, 6 h), EGTA (5 mmol/L, 1 min) or PTX (100 ng/mL, 24 h), and then treated with 100 μmol/L of αLA. Percentages of the αLA-induced Ca^{2+} response compared with digitonin are shown in (B) and (D). The data are presented as the mean±SEM of 3 independent experiments. $^bP<0.05$, $^cP<0.01$ versus non-treated cells.

increases are presumably mediated through FFA4[37].

Several studies have reported long-chain fatty acid-induced FFA4 activation in exogenously FFA4-expressing HEK293 cells[13, 15, 22, 24]. In these systems, FFA4-$G_{\alpha 16}$ fusion, FFA4-YFP fusion, SRE reporter, or $G_{\alpha 16GUST44}$ constructs were used to detect FFA4 activation, which is highly sensitive and convenient[13, 15, 22, 24]. However, the effects of free fatty acids on cells endogenously expressing FFA4 differ from those in overex-

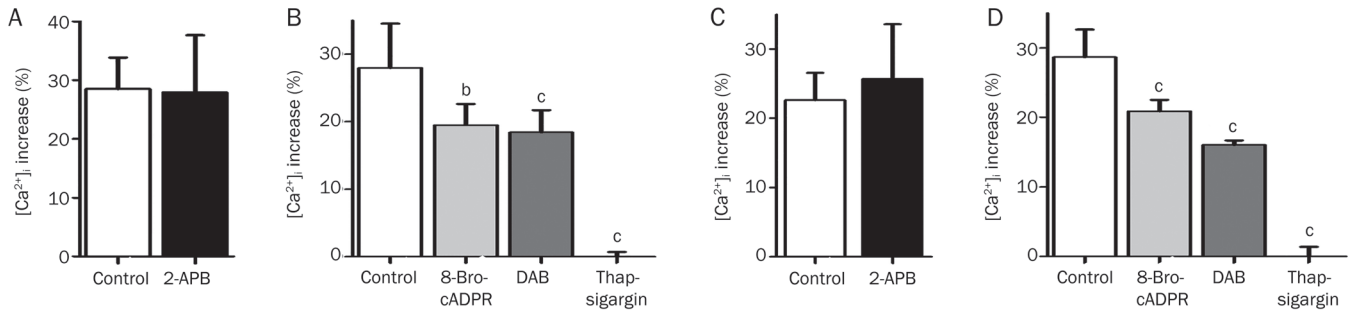

Figure 4. Effects of 2-APB, 8-bro-cADPR, DAB or thapsigargin on the αLA-induced Ca^{2+} responses in human colon epithelial cells. In (A) and (C), HCT116 and HT-29 cells were pretreated with vehicle or 2-APB (100 μmol/L, 15 min), then treated with 50 or 100 μmol/L αLA. In (B) and (D), HCT116 and HT-29 cells were pretreated with vehicle, 8-bro-cADPR (100 μmol/L, 20 min), DAB (1 μmol/L, 30 min) or thapsigargin (1 μmol/L, 30 min), then treated with 50 or 100 μmol/L αLA. Percentage of the αLA-induced Ca^{2+} response compared with digitonin. The data are presented as the mean±SEM of three independent experiments. $^bP<0.05$, $^cP<0.01$ versus non-treated cells.

pression systems. In FFA4-overexpressing systems, saturated fatty acids activated FFA4 but to a lesser extent than did unsaturated long-chain fatty acids[13]. However, in cells endogenously expressing FFA4, saturated long-chain fatty acids did not induce cellular responses in STC-1 cells or RAW264.7 macrophages[13, 15]; thus, FFA4 is considered a GPCR for ω-3 PUFA.

To determine the pharmacological relevance of individual nutrient fatty acids acting through FFA4, the ligand specificity of FFA4 in endogenously FFA4 expressing cells should be assessed. However, to date, αLA or GW9508 (a dual agonist for FFA1 and FFA4) have been typically used[15, 29, 37]. In the present study, long-chain fatty acids, including ω-3 PUFAs (αLA and EPA) and ω-6 PUFAs (linoleic acid and γ-linoleic acid), increased $[Ca^{2+}]_i$ with the same potency in human colonic epithelial cells. However, saturated fatty acids and monounsaturated fatty acids were inactive. Further, FFA4 was not specific for ω-3 PUFAs but recognized PUFAs with 18–20 carbons and more than 2 unsaturations. The present results using colonic epithelial cells are consistent with those previously obtained using cells endogenously expressing FFA4, such as STC-1 cells and RAW264.7 macrophages[13, 15]. This discrepancy between exogenous FFA4 and endogenous FFA4 was also reported by Hudson *et al*[24].

DHA did not induce a Ca^{2+} response in colonic cells but did do so in cells exogenously or endogenously expressing FFA4[13, 15]. Further, DHA induced β-arrestin recruitment in HCT116 and HT-29 cells. These results might be due to long-form FFA4 because in an ectopic expression system, long-form FFA4 induced β-arrestin-mediated responses but not G protein-mediated Ca^{2+} responses[21]. HCT116 cells express only long-form FFA4, whereas HT-29 cells express both isoforms. In addition, TUG891 less potently induced Ca^{2+} mobilization compared with other natural fatty acids, including αLA, in HT-29 and HCT116 cells. Hudson *et al* demonstrated that TUG891 was more potent than αLA in terms of Ca^{2+} mobilization in cells ectopically or endogenously expressing FFA4[24]. This discrepancy was also observed in RAW264.7 macrophages, which endogenously express FFA4[15, 24]. DHA-induced inhibition of TNFα production in macrophages was much greater than those of αLA or TUG891[24].

FFA4-mediated Ca^{2+} signaling has received little attention in cells endogenously expressing FFA4. In a previous study, Caco-2 intestinal cells, which express FFA4 but not FFA1, were used as endogenous FFA4-expressing cells[38], and DHA, EPA, and arachidonic acid enhanced Ca^{2+} cytosolic concentrations with similar efficiencies but different kinetics[38]. STC-1 cells (a mouse enteroendocrine cell line of intestinal origin) endogenously express FFA4 and FFA1, and in these cells, αLA-induced $[Ca^{2+}]_i$ increases were dependent on FFA4 and independent of FFA1[13]. Furthermore, αLA-mediated GLP-1 secretion by STC-1 cells was significantly inhibited by Ca^{2+}-free medium, implying that Ca^{2+} influx drives FFA4-mediated GLP-1 secretion[13]. However, nifedipine (an L-type Ca^{2+} channel blocker), thapsigargin, and U-73122 (a phospholipase C inhibitor) did not exhibit any inhibitory effect on GLP-1 secretion[13]. In contrast, the αLA-mediated CCK secretion through FFA4 in the same STC-1 cells was dependent on Ca^{2+} influx, inhibited by nicardipine or H-89 (a protein kinase A inhibitor) and not dependent on thapsigargin-sensitive Ca^{2+} stores[23]. In the present study, in colonic epithelial cells, the Ca^{2+} response was dependent on phospholipase C activation and Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores as well as partially dependent on Ca^{2+} influx, which is in contrast to the findings observed for Ca^{2+} signaling in STC-1 cells.

The involvement of PTX-insensitive $G_{q/11}$ G proteins has been reported in STC-1 and 3T3-L1 cells[13, 15, 23], which is consistent with our observations in colonic cells. 2-APB only slightly inhibited increase of $[Ca^{2+}]_i$ at maximal Ca^{2+} elevation in ectopic FFA4 expression systems[21]. However, 2-APB had no inhibitory effect on Ca^{2+} elevation in STC-1 or 3T3-L1 cells, which endogenously express FFA4[13, 15], consistent with our observations. Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores and the involvement of cADPR were shown for the first time to be components of FFA4 signaling. cADP-ribose activates Ca^{2+} release from the sarcoplasmic reticulum or ER through ryanodine receptors[39]. CD38, an ADP-ribosyl cyclase, is a 45 kDa type II transmembrane multifunctional enzyme that is involved in the formation of cADP-ribose in

mammals[39]. The activation of CD38 and subsequent production of cADP-ribose have been observed in GPCR signaling, such as that of the GPCRs for angiotensin II in the kidney[40], oxytocin in the hypothalamus[41], thromboxane in vascular smooth muscle cells[42] and acetylcholine in adipocytes[43]. Therefore, it would be interesting to investigate how FFA4 activates CD38 to produce cADP-ribose in colonic epithelial cells.

In summary, Ca^{2+} signaling through endogenous FFA4 in colonic epithelial cells exhibited specificity for long-chain polyunsaturated fatty acids with 18–20 carbon atoms and more than 2 unsaturations, which differs from the fatty acid requirements observed in cell exogenously overexpressing FFA4. In particular, DHA did not induce Ca^{2+} signaling but did induce β-arrestin recruitment. Further, Ca^{2+} signaling was composed of Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} influx, which are dependent on phospholipase C and cADP-ribose (Figure 5).

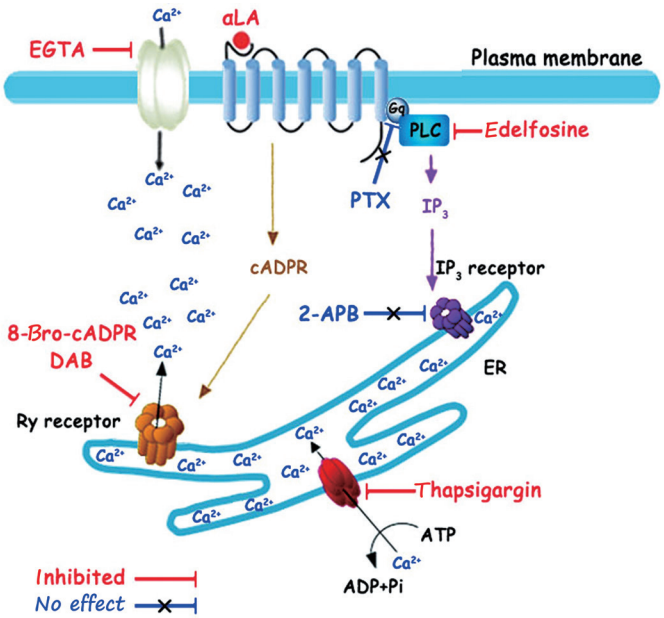

Figure 5. Schematic illustration of Ca^{2+} signaling mechanisms in colon epithelial cells.

Acknowledgements

This research was supported by the Korean National Research Foundation, which is funded by the Korean government (MSIP) (Grant No 2009-0083538).

Author contribution

Jung-min KIM and Dong-soon IM designed the study; Jung-min KIM, Soo-jin PARK, and Kyoung-pil LEE performed the experiments; and Jung-min KIM, Saeromi KANG, Jin HUANG, Jung-min LEE, Koichi SATO, Hae-young CHUNG, Fumikazu OKAJIMA, and Dong-soon IM participated in the data analysis and drafting of the manuscript.

Abbreviations

αLA, α-linolenic acid; GPCR, G protein-coupled receptor; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; FFA, free fatty acid; FFA1, free fatty acid receptor 1; FFA4, free fatty acid receptor 4; LCFA, long-chain fatty acid; 2-APB, 2-aminoethoxydiphenylborane; DAB, 2,2′-dihydroxyazobenzene; PTX, pertussis toxin; 8-bro-cADPR, 8-bromo-cyclic adenosine diphosphate ribose.

References

1 Im DS. Omega-3 fatty acids in anti-inflammation (pro-resolution) and GPCRs. Prog Lipid Res 2012; 51: 232–7.
2 Dyerberg J, Bang HO, Hjorne N. Fatty acid composition of the plasma lipids in Greenland Eskimos. Am J Clin Nutr 1975; 28: 958–66.
3 Hoy SM, Keating GM. Omega-3 ethylester concentrate: a review of its use in secondary prevention post-myocardial infarction and the treatment of hypertriglyceridaemia. Drugs 2009; 69: 1077–105.
4 Davidson MH, Stein EA, Bays HE, Maki KC, Doyle RT, Shalwitz RA, *et al.* Efficacy and tolerability of adding prescription omega-3 fatty acids 4 g/d to simvastatin 40 mg/d in hypertriglyceridemic patients: an 8-week, randomized, double-blind, placebo-controlled study. Clin Ther 2007; 29: 1354–67.
5 Zarepoor L, Lu JT, Zhang C, Wu W, Lepp D, Robinson L, *et al.* Dietary flaxseed intake exacerbates acute colonic mucosal injury and inflammation induced by dextran sodium sulfate. Am J Physiol Gastrointest Liver Physiol 2014; 306: G1042–55.
6 Matsunaga H, Hokari R, Kurihara C, Okada Y, Takebayashi K, Okudaira K, *et al.* Omega-3 fatty acids exacerbate DSS-induced colitis through decreased adiponectin in colonic subepithelial myofibroblasts. Inflamm Bowel Dis 2008; 14: 1348–57.
7 Camuesco D, Galvez J, Nieto A, Comalada M, Rodriguez-Cabezas ME, Concha A, *et al.* Dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, attenuates colonic inflammation in rats with DSS-induced colitis. J Nutr 2005; 135: 687–94.
8 Hassan A, Ibrahim A, Mbodji K, Coeffier M, Ziegler F, Bounoure F, *et al.* An α-linolenic acid-rich formula reduces oxidative stress and inflammation by regulating NF-κB in rats with TNBS-induced colitis. J Nutr 2010; 140: 1714–21.
9 Ibrahim A, Aziz M, Hassan A, Mbodji K, Collasse E, Coeffier M, *et al.* Dietary α-linolenic acid-rich formula reduces adhesion molecules in rats with experimental colitis. Nutrition 2012; 28: 799–802.
10 Lands WE. Biochemistry and physiology of n-3 fatty acids. Faseb J 1992; 6: 2530–6.
11 Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, *et al.* Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc Natl Acad Sci U S A 1997; 94: 4318–23.
12 Miyauchi S, Hirasawa A, Iga T, Liu N, Itsubo C, Sadakane K, *et al.* Distribution and regulation of protein expression of the free fatty acid receptor GPR120. Naunyn Schmiedebergs Arch Pharmacol 2009; 379: 427–34.
13 Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, *et al.* Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 2005; 11: 90–4.
14 Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, *et al.* Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature 2012; 483: 350–4.
15 Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, *et al.* GPR120 is an omega-3 fatty acid receptor mediating potent anti-

inflammatory and insulin-sensitizing effects. Cell 2010; 142: 687–98.
16 Davenport AP, Alexander SP, Sharman JL, Pawson AJ, Benson HE, Monaghan AE, *et al.* International union of basic and clinical pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands. Pharmacol Rev 2013; 65: 967–86.
17 Fredriksson R, Hoglund PJ, Gloriam DE, Lagerstrom MC, Schioth HB. Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives. FEBS Lett 2003; 554: 381–8.
18 Gotoh C, Hong YH, Iga T, Hishikawa D, Suzuki Y, Song SH, *et al.* The regulation of adipogenesis through GPR120. Biochem Biophys Res Commun 2007; 354: 591–7.
19 Burns RN, Moniri NH. Agonism with the omega-3 fatty acids α-linolenic acid and docosahexaenoic acid mediates phosphorylation of both the short and long isoforms of the human GPR120 receptor. Biochem Biophys Res Commun 2010; 396: 1030–5.
20 Moore K, Zhang Q, Murgolo N, Hosted T, Duffy R. Cloning, expression, and pharmacological characterization of the GPR120 free fatty acid receptor from cynomolgus monkey: comparison with human GPR120 splice variants. Comp Biochem Physiol B Biochem Mol Biol 2009; 154: 419–26.
21 Watson SJ, Brown AJ, Holliday ND. Differential signaling by splice variants of the human free fatty acid receptor GPR120. Mol Pharmacol 2012; 81: 631–42.
22 Galindo MM, Voigt N, Stein J, van Lengerich J, Raguse JD, Hofmann T, *et al.* G protein-coupled receptors in human fat taste perception. Chem Senses 2012; 37: 123–39.
23 Tanaka T, Katsuma S, Adachi T, Koshimizu TA, Hirasawa A, Tsujimoto G. Free fatty acids induce cholecystokinin secretion through GPR120. Naunyn Schmiedebergs Arch Pharmacol 2008; 377: 523–7.
24 Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E, *et al.* The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism. Mol Pharmacol 2013; 84: 710–25.
25 Sparks SM, Chen G, Collins JL, Danger D, Dock ST, Jayawickreme C, *et al.* Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). Bioorg Med Chem Lett 2014; 24: 3100–3.
26 Shimpukade B, Hudson BD, Hovgaard CK, Milligan G, Ulven T. Discovery of a potent and selective GPR120 agonist. J Med Chem 2012; 55: 4511–5.
27 Suzuki T, Igari S, Hirasawa A, Hata M, Ishiguro M, Fujieda H, *et al.* Identification of G protein-coupled receptor 120-selective agonists derived from PPARγ agonists. J Med Chem 2008; 51: 7640–4.
28 Hara T, Hirasawa A, Sun Q, Sadakane K, Itsubo C, Iga T, *et al.* Novel selective ligands for free fatty acid receptors GPR120 and GPR40. Naunyn Schmiedebergs Arch Pharmacol 2009; 380: 247–55.
29 Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, *et al.* Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 2006; 148: 619–28.
30 Talukdar S, Olefsky JM, Osborn O. Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases. Trends Pharmacol Sci 2011; 32: 543–50.
31 Arafa el SA, Abdelazeem AH, Arab HH, Omar HA. OSU-CG5, a novel energy restriction mimetic agent, targets human colorectal cancer cells *in vitro*. Acta Pharmacol Sin 2014; 35: 394–400.
32 Park SJ, Lee KP, Im DS. Action and signaling of lysophosphatidylethanolamine in MDA-MB-231 breast cancer cells. Biomol Ther (Seoul) 2014; 22: 129–35.
33 Wei JC, Meng FD, Qu K, Wang ZX, Wu QF, Zhang LQ, *et al.* Sorafenib inhibits proliferation and invasion of human hepatocellular carcinoma cells via up-regulation of p53 and suppressing FoxM1. Acta Pharmacol Sin 2015; 36: 241–51.
34 Ji XK, Xie YK, Zhong JQ, Xu QG, Zeng QQ, Wang Y, *et al.* GSK-3β suppresses the proliferation of rat hepatic oval cells through modulating Wnt/β-catenin signaling pathway. Acta Pharmacol Sin 2015; 36: 334–42.
35 Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, *et al.* Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 2003; 422: 173–6.
36 Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, *et al.* The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 2003; 278: 11303–11.
37 Wu Q, Wang H, Zhao X, Shi Y, Jin M, Wan B, *et al.* Identification of G-protein-coupled receptor 120 as a tumor-promoting receptor that induces angiogenesis and migration in human colorectal carcinoma. Oncogene 2013; 32: 5541–50.
38 Mobraten K, Haug TM, Kleiveland CR, Lea T. Omega-3 and omega-6 PUFAs induce the same GPR120-mediated signalling events, but with different kinetics and intensity in Caco-2 cells. Lipids Health Dis 2013; 12: 101.
39 Zhao YJ, Zhu WJ, Wang XW, Zhang LH, Lee HC. Determinants of the membrane orientation of a calcium signaling enzyme CD38. Biochim Biophys Acta 2014. doi:10.1016/j.bbamcr.2014.10.028.
40 Kim SY, Park KH, Gul R, Jang KY, Kim UH. Role of kidney ADP-ribosyl cyclase in diabetic nephropathy. Am J Physiol Renal Physiol 2009; 296: F291–7.
41 Lopatina O, Liu HX, Amina S, Hashii M, Higashida H. Oxytocin-induced elevation of ADP-ribosyl cyclase activity, cyclic ADP-ribose or Ca^{2+} concentrations is involved in autoregulation of oxytocin secretion in the hypothalamus and posterior pituitary in male mice. Neuropharmacology 2010; 58: 50–5.
42 Moss NG, Vogel PA, Kopple TE, Arendshorst WJ. Thromboxane-induced renal vasoconstriction is mediated by the ADP-ribosyl cyclase CD38 and superoxide anion. Am J Physiol Renal Physiol 2013; 305: F830–8.
43 Turovsky EA, Turovskaya MV, Dolgacheva LP, Zinchenko VP, Dynnik VV. Acetylcholine promotes Ca^{2+} and NO-oscillations in adipocytes implicating Ca^{2+}→NO→cGMP→cADP-ribose→Ca^{2+} positive feedback loop — modulatory effects of norepinephrine and atrial natriuretic peptide. PLoS One 2013; 8: e63483.