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Differential requirements of arrestin-3 and clathrin for ligand-dependent and -independent internalization of human G protein-coupled receptor 40☆

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ABSTRACT

G protein-coupled receptor 40 (GPR40) is believed to be an attractive target to enhance insulin secretion in patients with type 2 diabetes. GPR40 has been found to couple to Gq protein, leading to the activation of phospholipase C and subsequent increases in the intracellular Ca^{2+} level. However, the underlying mechanisms that regulate the internalization and desensitization of GPR40 remain to be elucidated. In the present study, a construct of GPR40 fused with enhanced green fluorescent protein (EGFP) at its C-terminus was constructed for direct imaging of the localization and internalization of GPR40 by confocal microscopy. In stably transfected HEK-293 cells, GPR40 receptors underwent rapid agonist-induced internalization and constitutive ligand-independent internalization. Our data demonstrated that the agonist-mediated internalization of GPR40 was significantly blocked by hypertonic sucrose treatment and by siRNA mediated depletion of the heavy chain of clathrin. In contrast, constitutive GPR40 internalization was not affected by hypertonic sucrose or by knock-down of clathrin expression, but it was affected by treatment with methyl-β-cyclodextrin (MβCD) and nystatin. Furthermore, our results using an arrestin-3-EGFP redistribution assay and siRNA-mediated knock-down of arrestin-3 and GRK2 expression revealed that arrestin-3 and GRK2 play an essential role in the regulation of agonist-mediated GPR40 internalization, but are not involved in the regulation of constitutive GPR40 internalization. Additionally, our observation showed that upon activation by agonist, the internalized GPR40 receptors were rapidly recycled back to the plasma membrane via Rab4/Rab5 positive endosomes, whereas the constitutively internalized GPR40 receptors were recycled back to the cell surface through Rab5 positive endosomes. Because FFA receptors exhibit a high level of homology, our observations could be applicable to other members of this family.

1. Introduction

Free fatty acids (FFAs) are not only an important energy source, but also participate significantly in a variety of physiological processes, including immunological reactions [1] and insulin secretion [2]. G protein-coupled receptor 40 (GPR40) has been identified as a receptor for a range of medium- and long-chain saturated and unsaturated fatty acids by three research groups in 2003 [1,3,4], and therefore, has been designated as free fatty acid receptor 1 (FFA1) [3,5]. GPR40 is abundantly expressed in mouse, rat, and human pancreatic β-cells and has also been detected in the human brain and monocytes [1,3,4]. FFAs have been shown to promote glucose-stimulated insulin secretion (GSIS) via GPR40 from rat pancreatic islets and an insulinoma β-cell line, MIN6 [1]. Previous studies showed that siRNA-mediated knock-down of GPR40 expression or deletion of GPR40 led to a significant decrease in insulin secretion in the presence of fatty acids [6,7] and after a long-term high fat feeding [8], whereas the overexpression of GPR40 in pancreatic β-cells augmented insulin secretion and improved oral glucose tolerance in vivo [9]. Combined, these results suggest that GPR40 is directly responsible for the acute stimulatory effects of FFA on insulin secretion. GPR40 has therefore become an attractive potential therapeutic target for regulating insulin secretion and diabetes.

Abbreviations: LA, linoleic acid; LNA, linolenic acid; GPCR, G-protein coupled receptor; HEK, human embryonic kidney; cAMP, cyclic AMP; siRNA, small interfering RNA; shRNA, small hairpin RNA; Rab, ras-related GTP-binding protein; BSA, bovine serum albumin; GRKs, G protein coupled receptor kinase.

☆ Disclosure summary: The authors have nothing to disclose.

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It is generally accepted that the activation of GPR40 causes an increase in intracellular Ca^{2+} levels from the endoplasmic reticulum and L-type Ca^{2+} channels via a Gαq-dependent signaling pathway in both recombinant cell systems and endogenously expressing cells [1,7,10], although observations of pertussis toxin-sensitive, oleic acid-induced, GPR40-dependent proliferation in MDA-MB-231 cells and intracellular Ca^{2+} mobilization in bovine neutrophils have suggested that Gαi/o may be involved in GPR40 activation [10,11]. Studies with siRNA and knock-out mice demonstrated that the deletion of GPR40 abolished intracellular Ca^{2+} mobilization and led to intracellular accumulation of inositol phosphates [12,13]. GPR40 has been shown to activate ERK1/2 phosphorylation via the PKC pathway and EGFR transactivation in response to oleic acids in HEK cells [14], NIT-1 and MCF-7 cells [15,16], and mouse embryonic stem cells [17]. Little is known, however, about the underlying molecular mechanisms that regulate the agonist-mediated internalization of GPR40.

Rapid internalization of the agonist-activated receptor into the intracellular membrane compartments of target cell plays an important role in the regulation of GPCR signaling and desensitization [18]. Accumulating evidence, however, has suggested that some GPCRs can be internalized even without agonist stimulation and/or activity by a process known as constitutive internalization [19]. The functional roles, molecular mechanisms, and structural determinants of constitutive internalization of GPCRs remain to be elucidated. In preliminary experiments, a substantial proportion of intracellular GPR40 even in the absence of agonist was observed. We have now characterized the agonist-promoted and agonist-independent internalization and trafficking of GPR40 in HEK-293 cells. Our data revealed that GPR40 not only underwent rapid internalization in a dose- and time-dependent manner via the clathrin-coated pit pathway in response to agonist, but also trafficked constitutively through a clathrin- and arrestin-independent pathway. Because FFA receptors exhibit a high level of homology, our observations could be applicable to other members of this protein family.

2. Materials and methods

2.1. Materials

Cell culture media and fetal bovine serum were obtained from Hyclone (Beijing, China). Lipofectamine 2000, G418, OPTI-MEM and transferrin Alexa Fluor® 594 conjugate were purchased from Invitrogen (Carlsbad, CA). The pEGFP-N1, pmCherry-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories, Inc. (Palo Alto, CA), TAKARA (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. The membrane probe DiI, nuclear dye Hoechst33258, RIPA lysis buffer, and anti-α-tubulin antibody were obtained from Beyotime (Haimen, China). The anti-arrestin monoclonal antibody was from BD Biosciences Pharmingen (San Diego, CA). The monoclonal anti-FLAG M2-FITC antibody was purchased from Sigma (St. Louis, MO). The anti-phospho-ERK1/2, anti-ERK1/2, anti-actin and horseradish peroxidase-conjugated secondary antibodies were from Cell Signaling (Danvers, MA). The clathrin HC siRNA (h), anti-clathrin HC (TD.1) siRNA, anti-GRK2 antibody and anti-GRK5 antibody were from Santa Cruz.

2.2. Molecular cloning and plasmid construction

GPR40 (GenBank accession no. NM_005303.2) was cloned by PCR using human genomic DNA as a template with the following primers: 5′-AAGCTTGCCACCATGGACCTGCCCCCGCAGCTCTCC-3′ (forward) and 5′-GGTACCGTCTTCTGGGACTTGCCCCCTTGCGT-3′ (reverse). Amplification for the pCMV-Flag vector was performed with the following primers: 5′-AAGCTTATGGACCTGCCCCCGCAGCTCTCC-3′ (sense) and 5′-AGATCTTTACTTCTGGGACTTGCCCCCTTG-3′ (antisense). The PCR products were inserted into the *HindIII* and *Kpn I* sites of the pEGFP-N1 vector and the *HindIII* and *Bgl* sites of the pCMV-Flag vector. Rab4 (GenBank accession no. NM_004578.3) was amplified for the pmcherry-N1 vector using the sense primer 5′-CCCTCGAGCTATGTCCGAAACCTACGATTTTTTG-3′ and antisense primer 5′-CCCAAGCTTACAACCACACTCCTGAGCGTTCG-3′; Rab5 (GenBank accession no. NM_004162.4) was cloned using the sense primer 5′-CCCTCGAGCTATGGCTAGTCGAGGCGCAACAAG-3′ and antisense primer 5′-CCCAAGCTTGTTACTACAACACTGATTCCTG-3′. Dominant-negative Rabs (Rab GDP) which contain a single mutation (S22N in Rab4, S34N in Rab5), causing the resulting protein to be constitutively inactive [20], were constructed by overlap extension PCR. All constructs were sequenced to verify the correct sequences.

A shRNA fragment targeting the CDS of GRK2 was generated using a pair of primers: 5′-CCGGCGGCGGTACTTCTACCTGTTCCTCGAGGAACAGGTAGAAGTACCGCCGTTTTTG-3′ (forward primer) where the target sequence was underlined and 5′-AATTCAAAAACGGCGGTACTTCTACCTGTTCCTCGAGGAACAGGTAGAAGTACCGCCG-3′ (reverse primer) and cloned into the above plasmid as described in the TRC protocols (http://www.broadinstitute.org), and the resulting plasmid was designated as GRK2 shRNA. GRK5 shRNA was constructed with a forward primer: 5′-CCGGACGAGATGATAGAAACAGAATCTCGAGATTCTGTTTCTATCATCTCGTTTTTTG-3′ and 5′-AATTCAAAAAACGAGATGATAGAAACAGAATCTCGAGATTCTGTTTCTATCATCTCGT-3′ (reverse primer). The scramble shRNA plasmid was obtained from addgene 1864.

2.3. Cell culture and transfection

Human embryonic kidney cell line (HEK-293) and Hela cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 4 mM L-glutamine (Invitrogen). Plasmids were transfected or co-transfected into HEK-293 or Hela cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Selection for stable expression was initiated 48 h after transfection by adding G418 (800 μg/ml).

2.4. Synthesis of small interfering RNAs and siRNA transfection

Small interfering RNAs (siRNAs) for arrestin-2 and arrestin-3 were purchased as a SMART pool from Dharmacon RNA Technologies (Lafayette, CO). The clathrin HC siRNA was from Santa Cruz. The nonspecific siRNA VIII (5′-AAA CUC UAU CUG CAC GCU GAC-3′) was used as the control for all siRNA experiments. The arrestin and CHC siRNAs were transfected according to the manufacturer's instructions. Transfected cells were divided for use in various assays 48 h after transfection.

2.5. Small hairpin RNA lentivirus production and viral infection

Viral production and infection were performed following the TRC protocols (the RNAi Consortium, http://www.broadinstitute.org). Briefly, 293T cells were co-transfected with the above viral vector-based shRNA and/or GRK2/5 variant plasmids together with lentivirus packaging vectors pMD2.G (Addgene 12259) and psPAX2 (Addgene 12260) using X-tremeGENE (Roche). 48 h and 72 h after transfection, the culture supernatants containing the released viral particles were collected, filtered through 0.45 μm membranes (Millipore SCHVU01RE), and used freshly or stored at 4 °C for less than 2 days. For viral infection, cells were plated and cultured overnight, and viral supernatants were added with polybrene (Sigma AL-118) at a final concentration of 8 μg/ml. In most cases, the multiplicity of infection (MOI) was estimated to be between 0.5 and 2. After 6–10 h, the media were replaced with fresh viral-free medium to allow further growth until use. Stable knockdown cells were obtained by 3 μg/ml puromycin (Shanghai, Sangon Biotech) co-incubated for 2 weeks and further validated by Western-blot.