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β-arrestin Recruitment and Biased Agonism at Free Fatty Acid Receptor 1

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*Running title: *Biased agonism at FFAR1/GPR40*

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Background: FFAR1/GPR40 is a potential target to enhance insulin secretion in type 2 diabetes. Yet, knowledge of GPR40's pharmacobiology remains incomplete.
Results: GPR40 functions via both G protein- and β-arrestin-mediated mechanisms. Endogenous and synthetic ligands differentially engage these pathways to promote insulin secretion.
Conclusion: GPR40 is subject to functionally relevant biased agonism.
Significance: Biased agonism at GPR40 could be exploited for therapeutic purposes.

ABSTRACT

FFAR1/GPR40 is a seven-transmembrane domain receptor (7TMR) expressed in pancreatic β cells and activated by free fatty acids (FFAs). Pharmacological activation of GPR40 is a strategy under consideration to increase insulin secretion in type 2 diabetes (T2D). GPR40 is known to signal predominantly via the heterotrimeric G proteins $G_{q/11}$. However, 7TMRs can also activate functionally distinct G protein-independent signaling via β-arrestins. Further, G protein- and β-arrestin-based signaling can be differentially modulated by different ligands, thus eliciting ligand-specific responses ("biased agonism"). Whether GPR40 engages β-arrestin-dependent mechanisms and is subject to biased agonism is unknown. Using BRET-based biosensors for real-time monitoring of cell signaling in living cells, we detected a ligand-induced GPR40:β-arrestin interaction, with the synthetic GPR40 agonist TAK-875 being more effective than palmitate or oleate in recruiting β-arrestins 1 and 2. Conversely, TAK-875 acted as a partial agonist of $G_{q/11}$-dependent GPR40 signaling relative to both FFAs. Pharmacological blockade of G_q activity decreased FFA-induced insulin secretion. In contrast, knockdown or genetic ablation of β-arrestin 2 in an insulin-secreting cell line and mouse pancreatic islets, respectively, uniquely attenuated TAK-875's insulinotropic activity, thus providing functional validation of the biosensor data. Collectively, these data reveal that in addition to coupling to $G_{q/11}$, GPR40 is functionally linked to a β-arrestin 2-mediated insulinotropic signaling axis. These observations expose previously

unrecognized complexity for GPR40 signal transduction and may guide the development of biased agonists showing improved clinical profile in T2D.

INTRODUCTION

The free fatty-acid receptor 1/G protein-coupled receptor 40 (FFAR1/GPR40) is a cell-surface, seven-transmembrane domain receptor (7TMR) activated by medium-to-long chain free fatty acids (FFAs) (1,2). GPR40 is predominately expressed in insulin-secreting pancreatic β-cells and mediates part of the acute stimulatory effects of various FFAs on insulin secretion. However, GPR40 does not mediate the long-term lipotoxic effects of FFAs on β-cell function (3,4). Given that FFA stimulate insulin secretion only when glucose levels are elevated, GPR40 could be targeted to enhance insulin secretion in T2D without the risk of iatrogenic hypoglycemia. Accordingly, GPR40 is generating substantial interest as a therapeutic target to enhance insulin secretion in type 2 diabetes (T2D) (5-7). Yet, despite significant investment in the development of selective GPR40 agonists, our understanding of GPR40's pharmacobiology remains incomplete (8).

To date, the predominant view holds that GPR40 signals primarily via the heterotrimeric G protein $G_{q/11}$ and that its biological effects are mediated by downstream second messenger molecules, namely diacylglycerol (DAG) and inositol 1,4,5, trisphosphate (IP_3) as well as Ca^{2+} mobilization (9,10). Additionally, coupling of GPR40 to the G_s/PKA/cAMP pathway has also been reported (11,12). It is becoming increasingly apparent, however, that 7TMRs are more than mere bimodal molecular switches that signal via linear G protein-dependent transduction pathways. Indeed, 7TMRs can also engage functionally distinct, G protein-independent signaling mechanisms via the multifunctional adapter proteins β-arrestins 1 and 2. Once believed to solely mediate receptor desensitization and internalization, β-arrestins are now recognized as key signaling hubs acting downstream of 7TMRs. According to the concept of "biased agonism", binding of structurally/chemically different ligands to a given 7TMR can stabilize unique receptor conformations, each differentially modulating β-arrestin- and G protein-dependent signaling (13-15). As a result, different ligands may impart distinct signaling and biological attributes to a given receptor. By selectively engaging only part of a receptor's potential intracellular partners and signaling cascades, biased ligands may provide refined pharmacological specificity and therapeutic efficacy with fewer side effects.

Recently, Qian *et al*. (16) demonstrated that β-arrestin 2 is implicated in linoleate-induced internalization of GPR40. Whether β-arrestin 1 and/or 2 partake in GPR40-dependent signaling and whether this receptor is subject to biased agonism, however, is unknown. This question is of particular relevance to the pharmacotherapy of T2D as several synthetic agonists of GPR40 are under clinical development. Amongst them, TAK-875 demonstrated encouraging therapeutic potential in Phase I and II clinical trials (17,18) but was subsequently discontinued during Phase III trials due to hepatotoxicity (19). Given the important implications of biased agonism at this emerging drug target, we aimed to ascertain i) whether GPR40 engages β-arrestin-dependent signaling, and ii) if $G_{q/11}$- and β-arrestin-mediated signaling can be differentially modulated by distinct ligands. Our results provide the first account of a functionally relevant, ligand-biased interaction between β-arrestin 2 and GPR40 that is involved in regulating GPR40's insulinotropic activity.

EXPERIMENTAL PROCEDURES

Reagents, solutions and plasmids- Dulbecco's modified Eagle's medium (DMEM) was from Multicell, while FBS and RPMI-1640 were from Invitrogen (Burlington, ON, Canada). TAK-875 was purchased from Selleckchem (Houston, TX, USA); palmitate and oleate were obtained from Sigma (St. Louis, MO, USA). Murine GPR40 cDNA was purchased from OriGene (pCMV6-mGPR40; SKU MC212962). The GPR40-GFP10 fusion

construct was created by deleting the GPR40 stop codon in pCMV6-mGPR40 with subsequent downstream insertion of linker-GFP10 cDNA (all performed by GenScript, USA).

Cell culture- Human Embryonic Kidney (HEK)-293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). INS832/13 were cultured in RPMI-1640 medium supplemented with 11 mM glucose, 10% FBS, 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. Cells were maintained at 37°C with 5% CO_2.

Obelin Ca^{2+} flux assay- HEK-293T cells were plated at 0.5 x 10^6 cells/well in 6-well tissue culture plates. The following day, cells were co-transfected with 1 μg cDNA encoding the Ca^{2+} biosensor obelin and 100 ng of either pcDNA3.1 or GPR40 cDNA using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. After overnight (18 h) incubation, transfected HEK-293T were passed and plated at ~1.0 x 10^5 cells/well in poly-L-ornithine-coated 96-well white-walled microtitre plates. The following day, HEK-293T were washed twice with 200 uL HBSS (Invitrogen) supplement with 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 0.2% Glucose (pH 7.4) and then incubated for 2-3h with 1 μM coelenterazine CP (Biotium) in the dark. Agonist-induced intracellular Ca^{2+} flux is reflected by the magnitude of the bioluminescent signal emitted by the obelin biosensor, which was recorded before and every 0.3 seconds after agonist injection in the SpectraMax L Microplate reader (Molecular Devices) for a total of 62.5s. The indicated concentrations of palmitate, oleate and TAK-875 were pre-complexed to a fixed concentration (20 μM final) of fatty-acid free BSA at 37°C for 1 h. Control wells were stimulated with vehicle (20 μM fatty-acid free BSA and 0.1% vol./vol. DMSO (TAK-875 solvent) or 0.17% vol./vol. ethanol (palmitate solvent)). The area under the resulting curves (AUC) was calculated using Prism GraphPad 6.0 as a measure of total Ca^{2+} flux; AUC values were used to construct dose curves. Data were expressed as a percentage versus the maximal response, which was consistently obtained with 80 μM palmitate.

G_q activation biosensor assay- HEK-293T cells were plated at 0.5 x 10^6 cells/well in 6-well tissue culture plates. The following day, the three subunits of the multimolecular G_q activation biosensor (20 ng each of RLucII-$G\alpha_q$ and Gβ1 and 100 ng of GFP10-tagged Gγ1) and 100 ng of pcDNA3.1 or GPR40 cDNA was transfected using polyethylenimine 25-kDa linear (PEI; Polysciences, Warrington, PA, USA) (20) at a 3:1 uL PEI/μg DNA ratio. The total amount of DNA transfected in each well was consistently adjusted to 2 μg with salmon sperm DNA (Invitrogen). At 48 h post-transfection, HEK-293T were washed once with Tyrode's buffer (140 mM NaCl, 1 mM $CaCl_2$, 2.7 mM KCl, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 5.6 mM glucose, 12 mM $NaHCO_3$, and 25 mM HEPES, pH 7.5), detached and plated in poly-L-ornithine-coated 96-well white-walled plates (~0.2 x 10^6 cells/well). Cells were allowed to adhere for 3h at 37°C, after which time vehicle or BSA-complexed GPR40 agonists (as for obelin Ca^{2+} flux assay above) were added to cells for 5 min at 37°C. Coelenterazine 400A (Biotium) was then added to a final concentration of 5 uM in Tyrode's buffer for 5 min. Readings were subsequently collected with a Mithras LB 940 multidetector plate reader (Berthold Technologies), allowing the sequential integration of the signals detected at 410 ± 40 nm (RLucII light emission) and 515 ± 15 nm (GFP10 light emission). The BRET signal was calculated as the ratio of the GFP10 light emission to RLucII light emission. Values are expressed as Net BRET by first subtracting vehicle-induced BRET from ligand-induced BRET (i.e., delta BRET) and then subtracting the delta BRET values calculated for cells transfected with pcDNA3.1 from the delta BRET values obtained in cells transfected with GPR40.

β-arrestin recruitment biosensor assays- HEK-293T cells were seeded as for obelin and G_q activity biosensor experiments. Cells were co-transfected (using linear polyethyleimine 25 KDa (PEI)) with 12.5 ng of either β-arrestin 1-RlucII or β-arrestin-2-RlucII and 500 ng of pcDNA3.1 or GPR40-

GFP10 cDNA. The total amount of DNA transfected in each well was always adjusted to 2 μg. After 48 h, cells were washed twice with Tyrode's buffer, detached and plated in poly-L-ornithine-coated 96-well microplates (~0.2 x 10^6 cells/well). Cells were allowed to adhere for 3h at 37°C. Total fluorescence was subsequently recorded with FlexStation (Molecular Devices; excitation filter at 400 nm; emission filter at 510 nm) to ensure equal GPR40-GFP10 expression across different wells and plates. For experiments in which G_q activity was inhibited, cells were preincubated with 100 nM of UBO-QIC. Vehicle or BSA-complexed GPR40 agonists were then added to all wells and cells were incubated at 37°C in the dark for 10 min. Thereafter, coelenterazine 400a (coel-400a) (Biotium) was added to each well at a final concentration of 2.5 μM. Cells were incubated at 37°C in the dark for an additional 5 min, after which time ligand-induced BRET was recorded with the Mithras LB940 Multimode Microplate Reader (Berthold Technologies) equipped with the BRET400-GFP2/10 filter set (acceptor at 515 ± 20 nm; and donor at 400 ± 70 nm filters). Values are expressed as Net BRET by first subtracting vehicle-induced BRET from ligand-induced BRET (i.e., delta BRET) and then subtracting the delta BRET values calculated for cells transfected with pcDNA3.1 and either RlucII-linked β-arrestin 1 or 2 from the delta BRET values obtained in cells co-transfected with GPR40-GFP10 and the β-arrestin biosensors.

siRNA-mediated knockdown of β-arrestin 2 and analysis by Western blot- INS832/13 (~6x10^6) were electroporated with 350 pmol of either non-targeting (negative control) or anti-β-arrestin 2 siRNAs via nucleofection (Amaxa® Nucleofector®) and subsequently seeded in 24-well culture plates at ~0.4x10^6 cells/well (for insulin secretion) and in 12-well plates at 1.2x10^6 cells/well (for protein extraction). Medium was replaced the day following nucleofection. Protein extraction and immunoblotting were performed as described (21) using an anti-β-arrestin 2 monoclonal antibody (Cell Signaling, clone C16D9; diluted 1:1000 in 5% non-fat milk/TBST). Membranes were subsequently stripped using Re-Blot Plus stripping solution (Millipore) and re-probed for α-Tubulin (Abcam).

Static insulin secretions in INS832/13- Electroporated cells were seeded as described above. For experiments with UBO-QIC, cells were seeded in 24-well culture plates at ~0.25x10^6 cells/well At ~48h post seeding, cells were starved for 2 h in RPMI-1640 medium supplemented with 1% FBS and 1 mM glucose, followed by a wash and 1 h incubation in KRB supplemented with 1 mM glucose and 0.1% BSA. For UBO-QIC experiments, 100 nM of the inhibitor was added 30 min into the 1 h KRB incubation. Cells were treated with the agents indicated in the text in KRB and insulin secretion was assayed following a 1 h static incubation. Insulin content was extracted with acid–alcohol. Secreted and intracellular insulin levels were measured using a rat insulin RIA kit (Millipore, St Charles, MO, USA). Each experimental condition was performed in triplicate.

Animals, islet isolations and static insulin secretions- Pancreatic islets were isolated from whole-body β-arrestin $2^{-/-}$ mice or WT littermates as described in (22). The original heterozygous β-arrestin $2^{+/-}$ mice were from R. J. Lefkowitz (Duke University Medical Center, Durham, NC, USA; described in (23). All animal studies complied with the authorization of the Ministry of Agriculture, France (D34-172-13). Islets were hand-picked after collagenase digestion of the pancreas and maintained overnight in RPMI-1640 supplemented with 7.5% FBS and 10 mM glucose. The following day, insulin secretion was assessed in 1-h static incubations using batches of 10 islets in bicarbonate KRB supplemented with 2.8 mM or 16.7 mM glucose with or without TAK-875 or palmitate. Both TAK-875 and palmitate were complexed for 1 h at 37°C with fatty-acid-free BSA to a final molar ratio of 1:5 as previously described (9). Control conditions contained an equal amount of BSA and vehicle (50% [vol./vol.] ethanol). Secreted and intracellular insulin levels were measured using a rat insulin RIA kit (Millipore, St Charles, MO, USA).

Data analysis- Results were analyzed using GraphPad Prism 6.0 (GraphPad Software) and are presented as mean ± SEM. Dose-response curves were fitted using the log(agonist) vs. response function (four parameters). All ligand concentrations shown (including EC_{50} values) correspond to those used for complexing to BSA and not free (uncomplexed) fractions. Statistical significance between was determined via unpaired Student's *t*-test or ANOVA (with Tukey's post hoc adjustment for multiple comparisons) as appropriate. $p<0.05$ was considered significant.

RESULTS

TAK-875 and FFAs promote β-arrestin recruitment to GPR40 with different efficacies- Recent work by Qian *et al*. (16) demonstrated that β-arrestin 2 is recruited to GPR40 in response to the FFA linoleate. Yet, it remains unknown whether different GPR40 ligands exhibit qualitative differences in the β-arrestin isoform they recruit and if such ligands are equi-efficacious in recruiting β-arrestins to the receptor. Thus, as a first step in establishing the existence of possible biased agonism at GPR40, we compared the potencies and efficacies of three distinct GPR40 agonists for β-arrestin 1 and 2 recruitment: the saturated and monounsaturated FFAs palmitate (PA; (C16:0)) and oleate (OA: (C18:1)), respectively, as well as the synthetic GPR40 agonist TAK-875. PA and OA were selected as the two most abundant circulating FAs in humans (24). The choice of TAK-875 was justified by its high selectivity for GPR40 vs. other fatty acid receptors (5), its lack of GPR40-independent insulinotropic action (25), and its overall structural dissimilarity to endogenous GPR40 ligands. Ligand-induced engagement of each β-arrestin by GFP10-tagged GPR40 was determined using a bioluminescence resonance energy transfer (BRET)-based assay that enables real-time monitoring of protein-protein interactions in living cells. These experiments were performed in HEK-293T cells, which lack endogenous GPR40 expression (26).

PA and OA (conjugated to a fixed concentration of 20 μM BSA) dose-dependently promoted the recruitment of β-arrestins 1 and 2 to GPR40 (Fig. 1A-B) with EC_{50} values in the low μM range (β-arrestin 1: 43.7 μM for PA; 51.7 μM for OA; β-arrestin 2: 42.4 μM for PA; 58.4 μM for OA). The synthetic GPR40 ligand TAK-875 (conjugated to a fixed concentration of 20 μM BSA) was much more potent (EC_{50}: 64.1 nM for β-arrestin 1; 54.7 nM for β-arrestin 2) and efficacious in promoting β-arrestin recruitment to GPR40. At its maximally effective concentration of 100 μM, the efficacy of PA for β-arrestin 1 and 2 recruitment was 38 ± 4% and 52 ± 7%, respectively, of that of 10 μM TAK-875. Similarly, the maximal effect of OA (150 μM) on β-arrestin 1 and 2 was 51 ± 4% and 55 ± 7%, respectively, of that of 10 μM TAK-875. These findings establish that relative to the synthetic GPR40 agonist TAK-875, the two endogenous GPR40 agonists PA and OA behave as a partial agonist for the recruitment of β-arrestins.

TAK-875 and FFAs exhibit distinct signaling signatures and biased agonism at GPR40- Having established that TAK-875 is more efficacious than either PA or OA at promoting β-arrestin 1 and 2 coupling to GPR40, we next determined if these ligands exhibit similar relative efficacies for activation of heterotrimeric G protein ($G_{q/11}$)-dependent signaling. We used a BRET-based biosensor to directly monitor agonist-induced activation of G_q. The G_q activation biosensor consists of an RLucII-tagged $G\alpha_q$ subunit, an untagged Gβ1 subunit and a GFP10-tagged Gγ1 subunit and monitors the physical separation of $G\alpha_q$ from Gβ/γ subunits upon agonist stimulation and receptor activation. Consequently, G protein activation results in a decreased BRET signal whose magnitude is tightly correlated to ligand efficacy (27,28). G_q activation was assayed in response to increasing concentrations of BSA-conjugated PA, OA or TAK-875. As shown in Fig. 2A, TAK-875 weakly activated G_q with an apparent EC_{50} around 60 nM. G_q activation by PA or OA

displayed no sign of saturation up to the highest testable concentrations (100 μM for PA and 150 μM for OA), due to insolubility (Fig. 2A). The maximal response produced by TAK-875 was 70 ± 4% of that observed with 100 μM PA and 58 ± 10% of that observed for 150 μM OA, thus demonstrating that relative to FFAs, TAK-875 is a partial agonist of GPR40-coupled $G_{q/11}$ activity. Analysis of downstream Ca^{2+} levels using the Ca^{2+} biosensor obelin corroborated the G_q activation data by showing that TAK-875 was 57 ± 7% and 66 ± 9% as effective as 80 μM PA and 100 μM OA, respectively, in increasing intracellular Ca^{2+} concentrations (Fig. 2B). Examination of $Ca^{2+}{}_i$ mobilization kinetics revealed that at maximally effective concentrations, all three ligands quickly (within 2 sec post-stimulation) triggered intracellular Ca^{2+} flux and elicited a maximal response at ~10s post-stimulation (Fig. 2B, inset).

These data establish that relative to the FFAs PA and OA, TAK-875 is a partial agonist of the G_q/Ca^{2+} signaling axis downstream of GPR40 and behaves as a β-arrestin-biased ligand. Conversely, the endogenous GPR40 ligands PA and OA act as $G_{q/11}$-biased GPR40 ligands by favoring $G_{q/11}$ activation over β-arrestin recruitment.

G_q activity is implicated in TAK-875 and FFA-induced β-arrestin recruitment to GPR40- It is now recognized that β-arrestin recruitment to 7TMRs can occur independently of G protein coupling (29). Interplay between these two processes, however, has also been described (30). To study the potential impact of GPR40-mediated G_q activation on β-arrestin recruitment, we assessed β-arrestin coupling to GPR40 in cells treated with a pharmacological inhibitor of G_q (UBO-QIC, 100 nM). Inhibition of G_q was confirmed using the G_q activation biosensor and was complete in all experiments (Fig. 3A). Inhibition of G_q reduced the potency of FFAs to recruit β-arrestins 1 and 2 to GPR40 (as evidenced by a rightward shift of the dose response curves). However, whereas the potency of TAK-875-induced β-arrestin recruitment to GPR40 was not affected by G_q inhibition, a clear reduction in maximal BRET was observed (48% and 45% reduction for β-arrestin 1 and β-arrestin 2, respectively) (Fig. 3B and C). These data imply that β-arrestin recruitment to GPR40 occurs via G_q-dependent and G_q-independent mechanisms. The different impact of G_q inhibition on TAK-875- and FFA-induced β-arrestin recruitment further highlights differences in how these two ligand classes engage β-arrestins.

TAK-875 and the FFA palmitate require different GPR40 downstream effectors for their maximal insulinotropic activity in INS832/13- Given GPR40's role in insulin secretion, we assessed the implication of β-arrestin 2 in mediating the insulinotropic action of TAK-875 and PA in insulin-secreting INS832/13 cells, which express GPR40. First, we measured insulin secretion in response to increasing concentrations of BSA-complexed TAK-875 in the presence of 11 mM glucose (Fig. 4A). At 10 μM, TAK-875 potentiated glucose-stimulated insulin secretion by 2.1-fold ($p<0.05$ vs. 11 mM glucose; n=3). Next, insulin secretion assays were conducted with TAK-875 and PA in β-arrestin 2 depleted INS832/13 cells. A ~50% knockdown of β-arrestin 2 protein levels (Fig. 4B) significantly reduced TAK-875-induced insulin secretion (52 ± 5% reduction vs. non-targeting negative control siRNA siNC1; $p<0.01$, n=3-6). Conversely, insulin secretion in response to PA was not significantly reduced by β-arrestin 2 knockdown (30 ± 17% reduction vs. siNC1; NS; Fig. 4C). In light of our previous observation linking GPR40-mediated G_q activation to β-arrestin recruitment, we investigated the impact of G_q inhibition on TAK-875- and PA-induced insulin secretion. Treatment of INS832/13 with the G_q inhibitor UBO-QIC (100 nM) did not significantly alter TAK-875's insulinotropic potential, while PA's effects were significantly reduced by 53 ± 11% (n=4, $p<0.05$; Fig. 4D). Collectively, these data indicate that the relative contributions of G_q and β-arrestin 2 activity favors G_q for the

insulinotropic response to PA but β-arrestin 2 for the response to TAK-875.

TAK-875-, but not FFA-induced insulin secretion is reduced in β-arrestin 2$^{-/-}$ mouse islets of Langerhans- To validate the insulinotropic function of the GPR40:β-arrestin 2 axis in a physiologically-relevant experimental system, insulin secretion was assessed *ex vivo* in 1-h static incubations of islets of Langerhans isolated from male β-arrestin 2$^{-/-}$ mice and their wild-type (WT) littermates. One hundred μM BSA-complexed TAK-875 significantly potentiated glucose-stimulated insulin secretion in WT islets ($p<0.01$ vs. 16.7 mM glucose; n=4-5; Fig. 5A) and this concentration was chosen for subsequent experiments. Treatment of WT and β-arrestin 2$^{-/-}$ islets with 16.7 mM glucose resulted in a similar induction of insulin secretion (Fig. 5B), as previously reported (22). PA or OA similarly potentiated glucose-stimulated insulin secretion from WT and β-arrestin 2$^{-/-}$ islets. In contrast, potentiation of glucose-stimulated insulin secretion by TAK-875 was significantly dampened in β-arrestin 2$^{-/-}$ islets (31 ± 6% reduction vs. WT, $p<0.001$; n=5-8). These results corroborate those obtained following siRNA-mediated knockdown of β-arrestin 2 in INS832/13 and provide key functional validation of the biosensor data. They show, in a physiologically relevant system, that TAK-875 promotes insulin secretion at least in part via a β-arrestin 2-dependent mechanism whereas FFAs insulinotropic activity is largely β-arrestin-independent, confirming the bias between these ligands.

DISCUSSION

The free fatty acid receptor GPR40 has emerged as a promising therapeutic target to enhance insulin secretion in T2D. Knowledge on GPR40-mediated signal transduction remains limited to classical notions of 7TMR biology, with the prevailing view holding that GPR40 primarily acts through G protein-dependent mechanisms (9,10,12). Unknown was this receptor's ability to function via "non-canonical" and mechanistically distinct β-arrestin-dependent (G protein-independent) mechanisms. We show here, using BRET-based molecular biosensors, that in addition to coupling to the $G_{q/11}/Ca^{2+}$ pathway, GPR40 interacts with β-arrestins 1 and 2 and is functionally linked to a β-arrestin 2-mediated signaling axis implicated in insulin secretion. Further, our findings identify ligand-specific signaling signatures downstream of GPR40, with the synthetic agonist TAK-875 acting as a β-arrestin-biased GPR40 agonist and the endogenous GPR40 agonists PA and OA being $G_{q/11}$-biased ligands. With these findings, we have established the existence of biased agonism at GPR40 (Fig. 6).

Biased agonism reportedly emanates from the ability of structurally/chemically distinct ligands of a given 7TMR to stabilize different receptor conformations (14,31) that may influence the receptor's affinity for various downstream signaling effectors. It is therefore conceivable that TAK-875-bound GPR40 adopts a conformation that displays higher affinity for the β-arrestin versus $G_{q/11}$ pathway, while the conformation(s) stabilized by PA and OA preferentially activate(s) $G_{q/11}$. Various studies assessing GPR40's interaction with different ligands (both endogenous and synthetic) have revealed the existence of multiple ligand-binding sites (32). Of particular interest, mutation of residues previously shown to be important for the binding of the GPR40 synthetic agonist GW9508 (33) influenced TAK-875-induced Ca^{2+} influx without significantly impacting the activity of the FFA gamma linolenic acid (γLA) (25). Mutation of two of the three residues shown to be indispensible for GPR40 activity (i.e., Arg183 and Arg258) rendered TAK-875 completely inactive in Ca^{2+} flux assays while only reducing the potency of γLA in this regard. Further, Huage *et al*. (12) described agonists promoting dual coupling of GPR40 to $G_{q/11}$ and G_s pathways and used radioligand binding studies to show that dual $G_{q/11}$ / G_s agonists bind GPR40 at a site distinct from that used by agonists stimulating $G_{q/11}$ signaling only. Finally, the recent crystallization of GPR40 co-complexed with

TAK-875 revealed the existence of three putative ligand binding sites and showed that TAK-875 exhibits a binding mode that is likely distinct from that for FFAs (34). Altogether, these results denote a substantial degree of ligand binding diversity and binding pocket plasticity at GPR40 that may translate into ligand-specific, functionally distinct receptor states. Still, direct proof that FFAs and TAK-875 exhibit unique binding modes and stabilize functionally different receptor conformations remains to be provided.

The demonstration of biased agonism at GPR40 was greatly enabled by our ability to directly assess GPR40-linked $G_{q/11}$ activation. Indeed, our study is the first to directly measure $G_{q/11}$ activation following ligand stimulation of native GPR40 in living cells. In a previous work, Yabuki *et al*. (25) used Ca^{2+} flux assays to define TAK-875 as a partial agonist of GPR40-coupled G_q activity. In our study, measures of G_q activation and Ca^{2+} flux were used in a complementary manner to directly demonstrate that relative to PA and OA, TAK-875 is a partial agonist of the G_q signaling axis (Fig. 2). Together, these independent but interrelated measures were instrumental in demonstrating the existence of biased agonism at GPR40.

To demonstrate that the biased agonism observed with biosensors in a heterologous expression system was functionally and physiologically relevant, we assessed insulin secretion in two experimental models: 1) the insulin-secreting cell line INS832/13 in which G_q activity was inhibited or following siRNA-mediated knockdown of β-arrestin 2, and 2) WT and β-arrestin $2^{-/-}$ islets of Langerhans. Based on the biosensor–derived data, we predicted that the blockade of G_q activity would primarily affect FFA-induced insulin release whereas the loss of β-arrestin 2 would impact TAK-875's secretagogue activity to a greater degree than that of FFAs. Indeed, this prediction proved to be correct, as shown in Fig. 4 and 5. These data are in agreement with previous data in mouse islets demonstrating that GPR40-dependent G_q signaling accounts for ~50% of FFA-induced potentiation of insulin secretion (35). Importantly, these data also establish a novel role for β-arrestin 2 in GPR40-mediated insulin secretion. Of note, TAK-875-induced insulin secretion was only partially affected by loss of β-arrestin 2, thus implying that β-arrestin 2-independent mechanisms are also operative in this regard. Such mechanisms may involve β-arrestin 1, which is also recruited to GPR40 and has been shown to interact with and mediate insulinotropic signaling downstream of two 7TMRs (i.e., the glucagon-like peptide-1 receptor and M3-muscarinic receptor) (36-38). β-arrestin recruitment to GPR40 was also detected in response to PA and OA; yet, potentiation of insulin secretion by these FFAs was insensitive to the ablation of β-arrestin 2 expression. These data may indicate that a minimal recruitment "threshold", which is unattained by PA- or OA-stimulated GPR40, must be reached for the production of a functional outcome. Alternatively, β-arrestins recruited in response to TAK-875 and FFAs may serve distinct functions. Indeed, recent immunofluorescence microscopy data from Qian *et al.* (16) demonstrated that the FFA linoleate results in β-arrestin 2-specific internalization of GPR40.

Our data have also unveiled cross talk between the G_q-dependent and β-arrestin-mediated signaling pathways downstream of GPR40. Interestingly, pharmacological inhibition of G_q activity differentially affected TAK-875- and FFA-induced β-arrestin recruitment to GPR40 (Fig. 3). Inactivation of G_q reduced the potency of FFAs to engage β-arrestins, while (in a manner comparable to carbachol-stimulated muscarinic acetylcholine receptor M1 (30)) it reduced the maximal BRET in response to TAK-875 without influencing potency. This combination of BRET outcomes is consistent with an alteration in the overall β-arrestin conformational landscape recruited to GPR40. β-arrestin recruitment to 7TMRs occurs via both G protein-dependent and -independent mechanisms. In the former, activation of the G protein results in the dissociation of Gα and Gβ/γ subunits. Consequently, Gβ/γ recruits G protein-coupled receptor kinase 2 (GRK2) to the receptor, which in turn phosphorylates

cytosolic residues on the 7TMR to promote β-arrestin recruitment (39). Additionally, β-arrestin recruitment can also be activated without G-protein coupling (40). This may explain why in our study, inactivation of G_q (which affects β-arrestin recruitment to GPR40) does not impact insulin secretion in response to a ligand whose insulinotropic effect is partly β-arrestin 2-dependent.

The discovery of β-arrestin-induced insulinotropic signaling and biased agonism at GPR40 may have important pharmacotherapeutic implications. Despite the sudden termination of TAK-875's clinical development during phase III trials, GPR40 remains a pharmacological target of interest for the treatment of T2D. Our data demonstrate that GPR40 signaling is neither linear nor entirely $G_{q/11}$-dependent which, together with the failure of TAK-875, highlight the importance of characterizing the full complexity of this 7TMR's signaling mechanisms. The existence of G protein-dependent and β-arrestin-mediated signaling pathways downstream of GPR40 opens the possibility of developing pathway-selective agonists that, through the fine differential regulation of both pathways, will afford maximal long-term therapeutic benefit and safety. β-arrestins have been shown to promote β-cell cytoprotection and survival via promoting ERK1/2-dependent phosphorylation of the anti-apoptotic factor Bad (36). Further, β-arrestins negatively regulate the production of pro-inflammatory cytokines by interfering with NF-κb signaling, a pathway implicated in β-cell dysfunction in T2D (41). Finally, β-arrestins have been implicated in compensatory β-cell proliferation and insulin signaling (22). It is therefore tempting to speculate that contrary to unbiased GPR40 agonists, which would simply act as insulin secretagogues, β-arrestin-biased GPR40 agonists may also afford additional cytoprotective, anti-inflammatory and mitogenic actions, although this needs to be directly tested.

In conclusion, our data expose unrecognized diversity and texture in GPR40 signal transduction and function. We have shown that GPR40-mediated insulinotropic signaling can occur via a $G_{q/11}$-dependent pathway as well as a novel β-arrestin 2-dependent axis. Further, these insulinotropic signaling axes are differentially engaged by different GPR40 ligands, with the endogenous ligands PA and OA preferentially activating $G_{q/11}$ signaling and the synthetic ligand TAK-875 promoting β-arrestin 2-mediated signal transduction. Such biased agonism at GPR40 may instigate the development of new pathway-selective GPR40 agonists showing improved clinical efficacy and safety in T2D.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

AM, JG, MB and VP conceived and coordinated the study and wrote the manuscript. AM, KV, EC and JG performed and analyzed the experiments shown in Figures 1-4 and 5A. GB performed and analyzed the experiments shown in Figure 5B. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1: The synthetic GPR40 agonist TAK-875 is more efficacious than the endogenous agonists palmitate or oleate in promoting β-arrestin recruitment to GPR40. HEK-293T cells transiently overexpressing GPR40-GFP10 and the β-arrestin 1 (A) or β-arrestin 2 (B) recruitment biosensor were treated with vehicle or increasing concentrations of BSA-complexed palmitate (10-100 μM), oleate (10-150 μM) or TAK-875 (0.01–10 μM). Agonist-induced recruitment of each β-arrestin was determined by measuring BRET at 15 min post-agonist stimulation. Data shown are mean ± SEM of 4 and 7 independent experiments for β-arrestin 1 and β-arrestin 2, respectively.

Figure 2: Relative to palmitate or oleate, TAK-875 is a partial agonist of heterotrimeric G protein ($G_{q/11}$)-dependent signaling downstream of GPR40. G_q pathway activation was determined in HEK-293T expressing pcDNA3.1 or GPR40 with the G_q activation biosensor (A) or the intracellular [Ca^{2+}] biosensor obelin (B). (A) Transiently-transfected cells were stimulated with vehicle or increasing concentrations of either BSA-conjugated palmitate (10-100 μM), oleate (10-150 μM) or TAK-875 (0.1-30 μM) for 5 min, after which time BRET between $G\alpha_q$-RlucII and GFP10-Gγ1 was measured. Activation of G_q, which results in the physical dissociation of $G\alpha_q$ and Gβγ subunits, is represented by a decreased BRET signal the magnitude of which is directly correlated to G_q activation. Data are mean ± SEM of 5-9 independent determinations. (B) Cytosolic [Ca^{2+}] was assessed following stimulation of transfected cells with 10-100 μM palmitate, 10-100 μM oleate or 0.1-10 μM TAK-875. Inset: Intracellular Ca^{2+} flux kinetics immediately following stimulation of transfected HEK-293T with maximally effective concentrations of BSA-conjugated palmitate (80 μM), oleate (100 μM) or TAK-875 (10 μM). Data presented are mean ± SEM of 8-10 independent experiments. Kinetics and dose-response data are derived from same experiments. N/D: non-determinable due to lack of saturation.

Figure 3: Pharmacological inhibition of G_q activity influnces β-arrestin recruitment to GPR40 in a ligand-specific manner. HEK-293T cells transiently overexpressing GPR40-GFP10 and either the G_q activation biosensor (A) or the β-arrestin 1 (B) or β-arrestin 2 (C) recruitment biosensors were pre-treated (30 min) with vehicle (Control) or with the G_q inhibitor UBO-QIC (100 nM). Cells transfected with β-arrestin recruitment biosenors were subsequently simulated with increasing concentrations of BSA-complexed palmitate (10-100 μM), oleate (10-150 μM) or BSA-complexed TAK-875 (0.01–10 μM). Agonist-induced recruitment of each β-arrestin was determined by measuring BRET at 15 min post-agonist stimulation (B-C). Data shown are mean ± SEM of 4-7 independent determinations. For each experiment, validation of G_q inactivation efficiency by UBO-QIC was performed in parallel using cells transfected with the G_q activation biosensor (A). ***, $p<0.001$ by unpaired Student's *t*-test.

Figure 4: The insulinotropic activity of TAK-875 and palmitate requires different GPR40 downstream effectors. (A) Determination of maximally effective insulinotropic dose of TAK-875. Insulin-secreting INS832/13 cells were treated with increasing concentrations of BSA-complexed TAK-875 (1 μM, 10 μM or 100 μM) in the presence of 11 mM glucose. Data are mean ± SEM of 3 independent experiments. *, $p<0.05$ vs. 11 mM glucose by one-way ANOVA. (B) Validation of β-arrestin 2 knockdown efficiency via Western blot. INS832/13 were electropoarted with either a non-targeting negative control siRNA (siNC1) or an anti-β-arrestin 2 siRNA (sibArr2). At 48h post-electroporation, immunoblotting was performed with an anti-β-arrestin-2 antibody or α- tubulin antibody (loading control). β-arrestin 2 knockdown efficiency is shown as percentage vs. siNC1 electroporated cells; all data are normalized by α-tubulin. The mean ± SEM of 4 independent experiments is shown. (C) INS832/13 electroporated with siNC1

or sibArr2 were stimulated with BSA-complexed TAK-875 (10 μM) or palmitate (500 μM) at 11 mM glucose. Secreted insulin was measured after 1-h via RIA. Data are normalized by total insulin content and represented as fold vs. siNC1-electroporated cells. The mean ± SEM of 3-7 independent determinations is displayed. (D) Insulin secretion was assessed in 1-h static incubations of INS832/13 with 11 mM glucose in the absence (-) or presence (+) of the Gq inhibitor UBO-QIC (100 nM). Data are normalized by total insulin content and represented as fold vs. non UBO-QIC-treated cells. Shown is the mean ± SEM of 4-5 independent determinations. ns, non-significant by unpaired Student's *t*-test; *, $p<0.05$ by unpaired Student's *t*-test; **, $p<0.01$ by unpaired Student's *t*-test.

Figure 5: β-arrestin 2 is implicated in insulinotropic action of TAK-875 but FFAs in pancreatic islets. (A) Determination of maximally effective insulinotropic dose of TAK-875. Pancreatic islets isolated from wild-type (WT) C57Bl/6 mice were stimulated with 10 μM or 100 μM of BSA-complexed TAK-875 in the presence of 16.7 mM glucose. Data are mean ± SEM of 4-5 independent determinations. **, $p<0.01$ vs. 16.7 mM glucose by one-way ANOVA. (B) Pancreatic islets isolated from WT or β-arrestin $2^{-/-}$ male C57Bl/6 mice were incubated with KRBH buffer containing 2.8 mM or 16.7 mM glucose in the absence or presence of BSA-complexed palmitate (500 μM), oleate (500 μM) or TAK-875 (100 μM). Insulin secretion was assessed in 1 h static incubations. The mean ± SEM of at least 5 independent experiments is shown. ***, $p<0.001$ by two-way ANOVA.

Figure 6: Hypothetical model for biased agonism at GPR40. Activation of GPR40 promotes insulin secretion via G protein ($G_{q/11}$)-dependent and β-arrestin-dependent mechanisms, with the endogenous GPR40 agonists palmitate (PA) and oleate (OA) and the synthetic agonist TAK-875 displaying converse relative efficacies for $G_{q/11}$ and β-arrestin pathway activation (as depicted by arrow thickness). *Left side*: TAK-875 acts as a partial agonist of $G_{q/11}$ activation. β-arrestin recruitment to GPR40 occurs via $G_{q/11}$-dependent and -independent mechanisms. *Right side*: The $G_{q/11}$ pathway serves as the main GPR40-downstream signaling pathway responsible for the insulinotropic activity of PA and OA. PA and OA also engage β-arrestins, but with lower efficacy than is observed with TAK-875. Activation of β-arrestins by PA and OA is not implicated in mediating their insulin secretagogue effect.

Figure 1

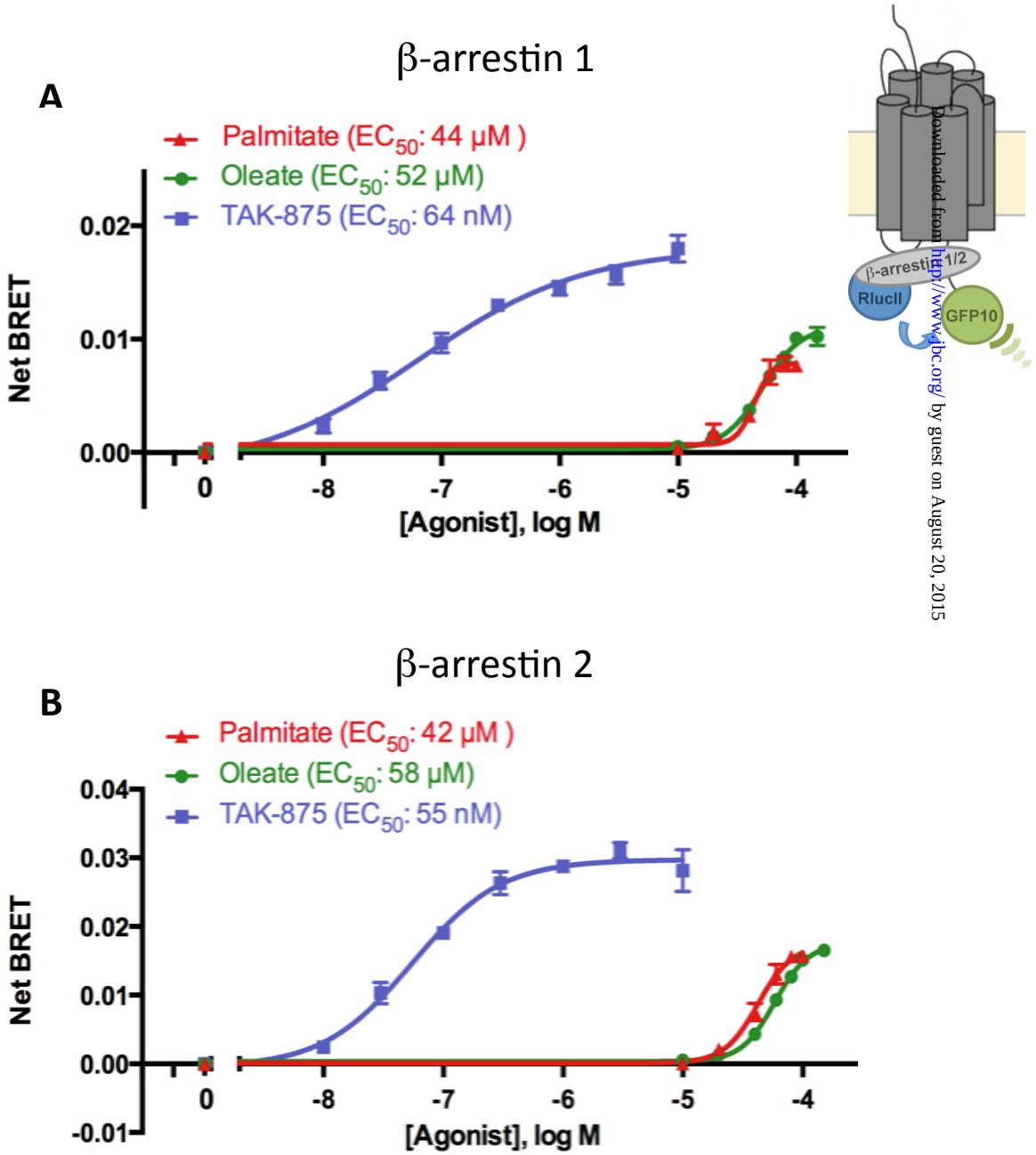


Figure 2

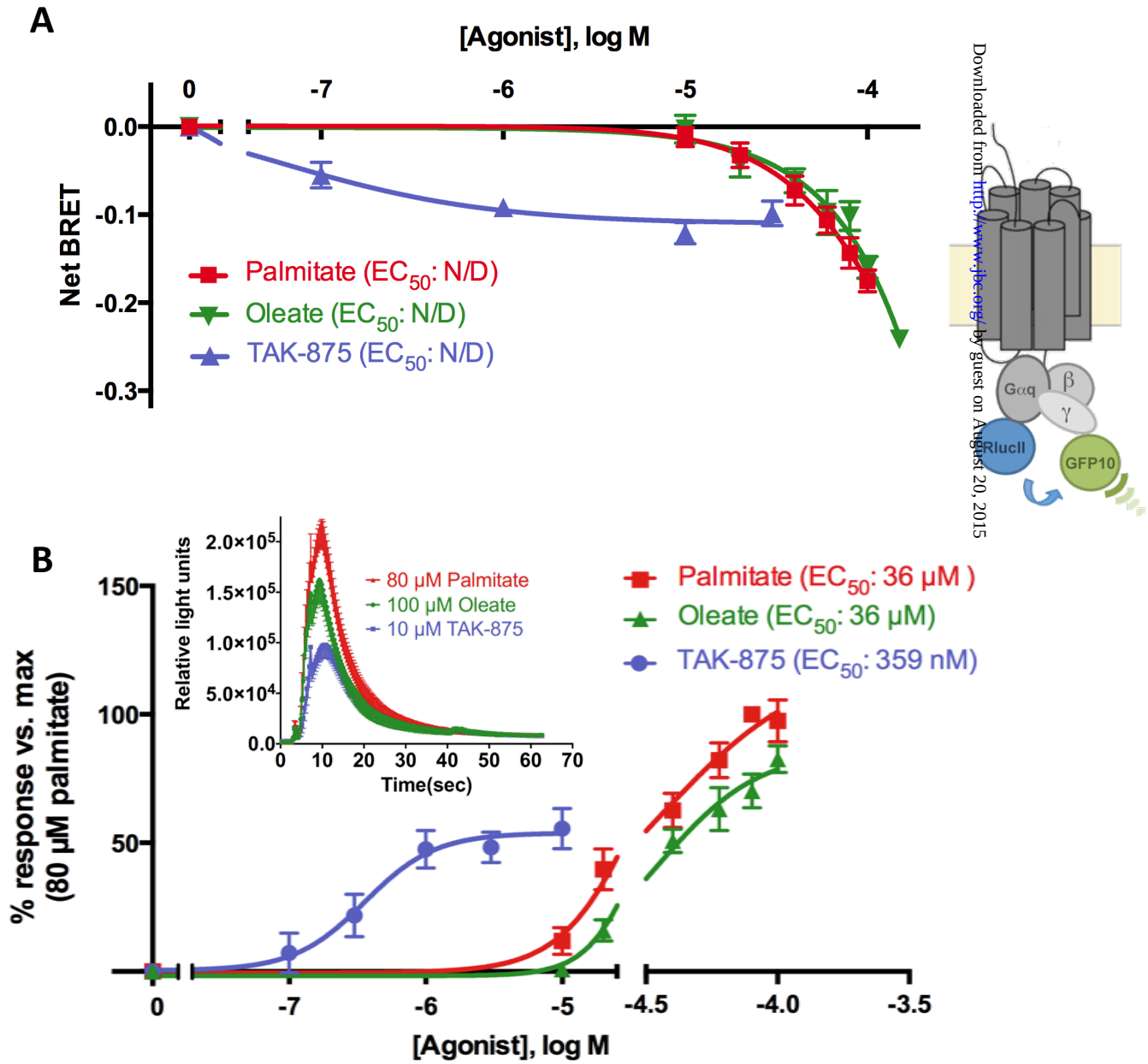


Figure 3

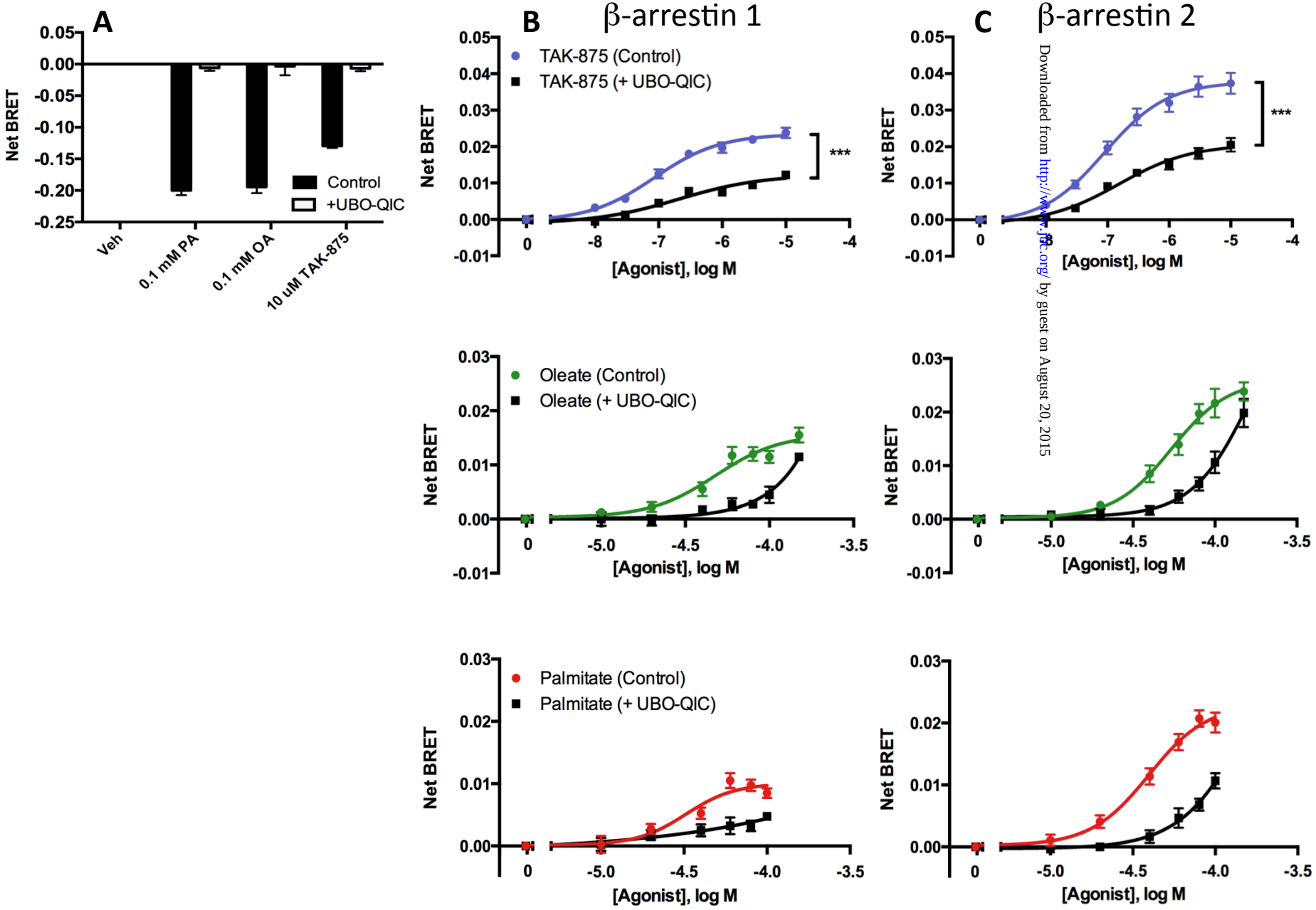


Figure 4

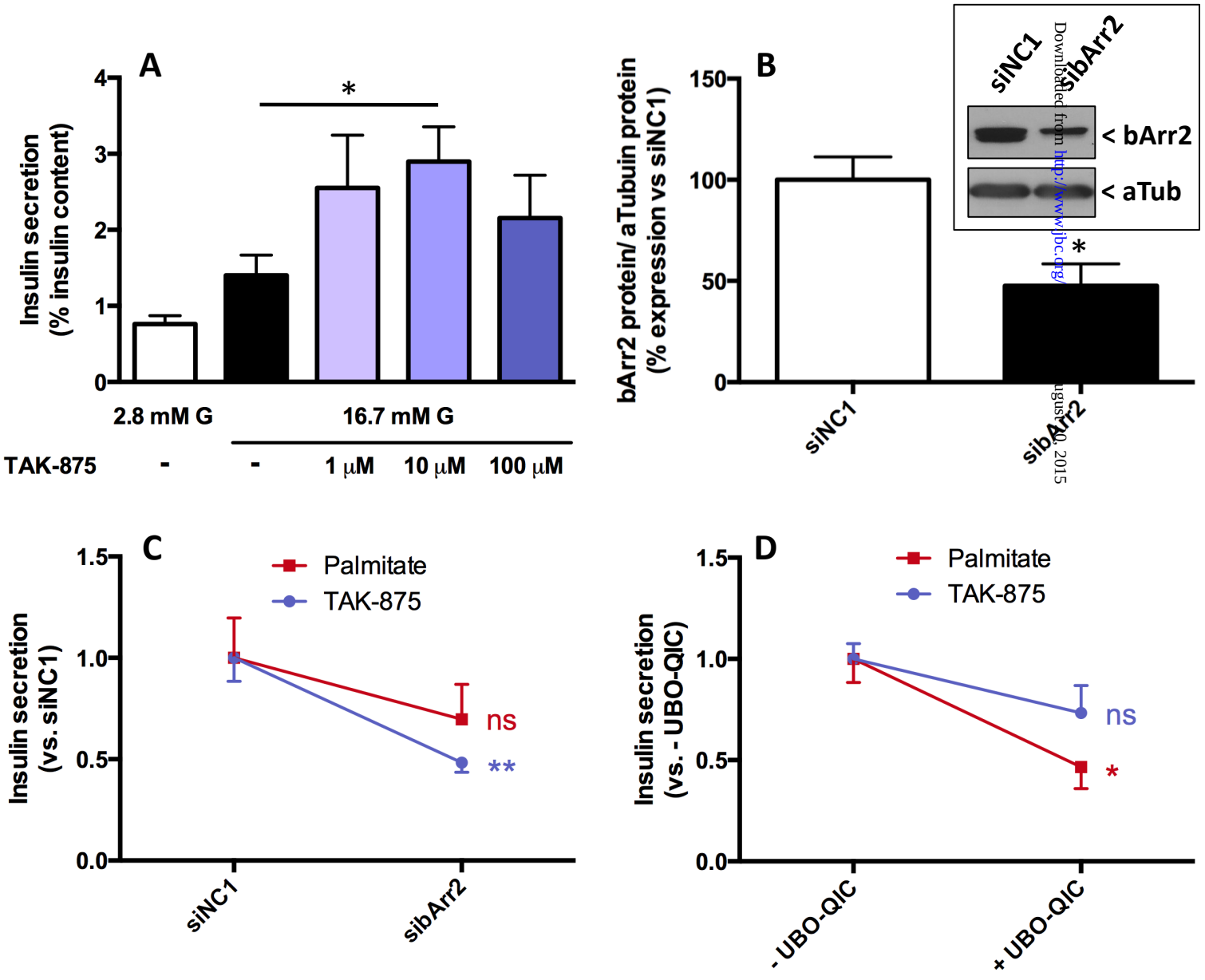


Figure 5

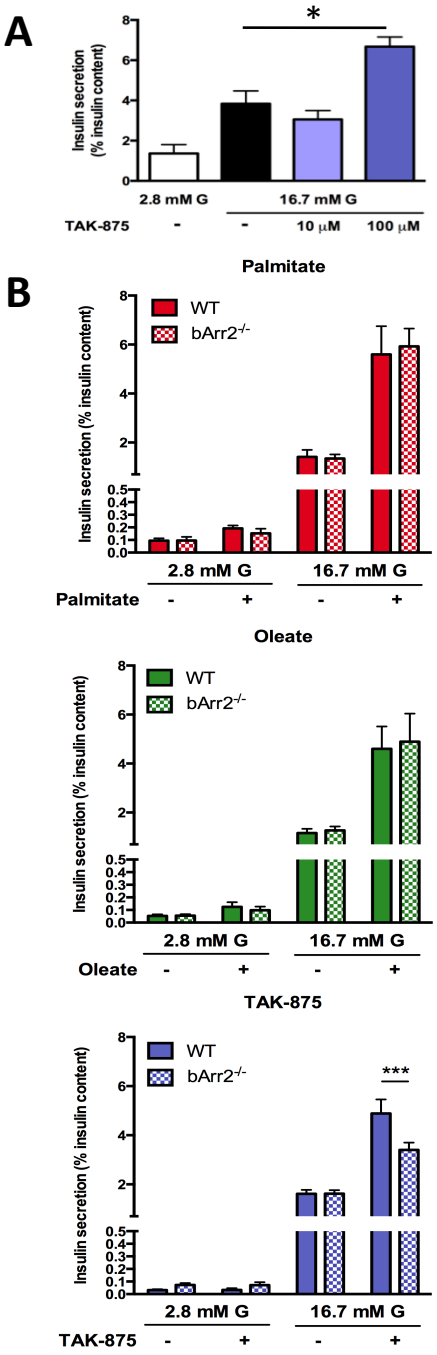
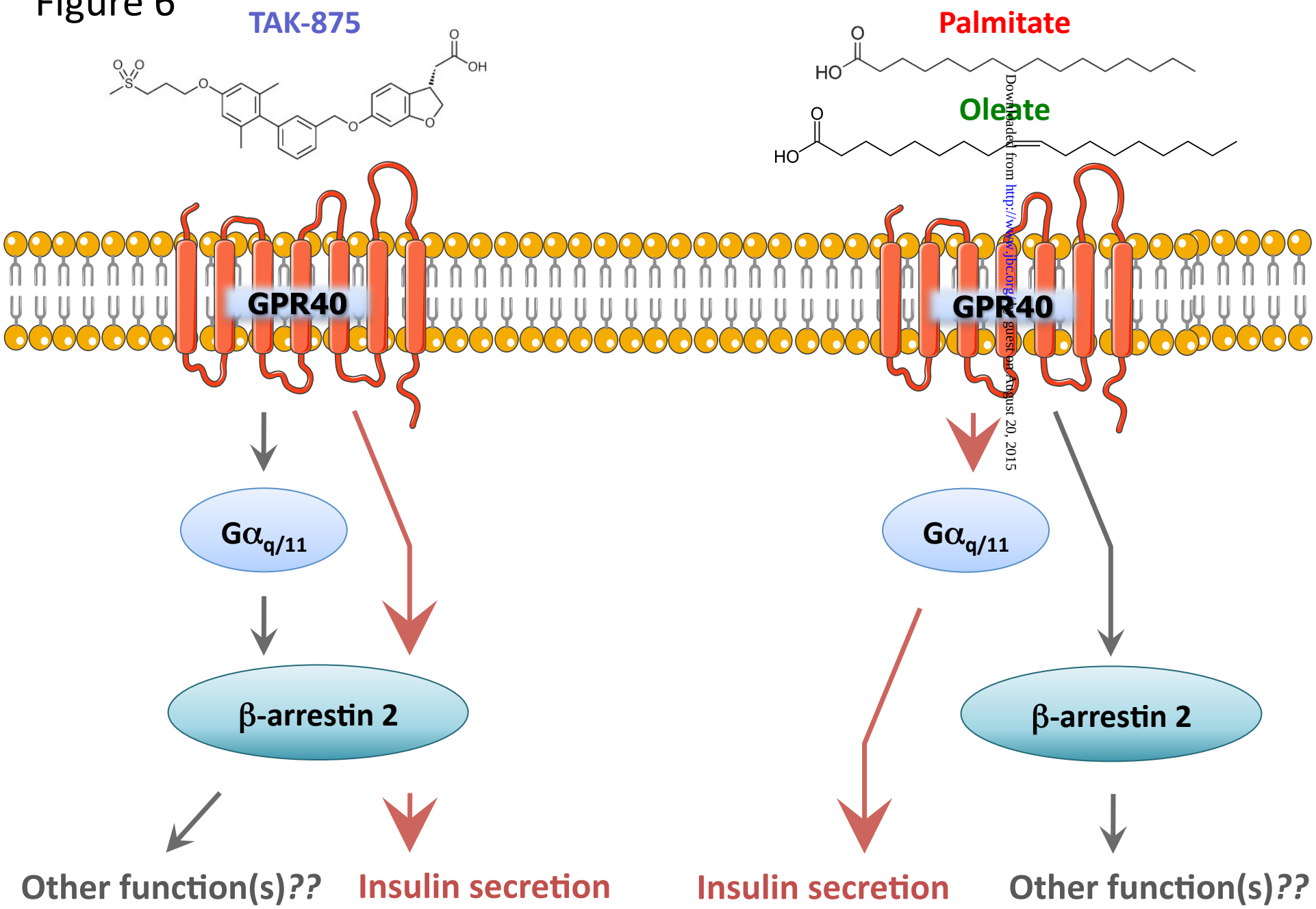


Figure 6



Signal Transduction:

β-arrestin recruitment and biased agonism at free fatty acid receptor 1

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