Nicotinic acid is one of the most effective agents for both lowering triglycerides and raising HDL. However, the side effect of cutaneous flushing severely limits patient compliance. As nicotinic acid stimulates the GPCR GPR109A and G_{i/o} G proteins, here we dissected the roles of G proteins and the adaptor proteins, \( \beta \)-arrestins, in nicotinic acid–induced signaling and physiological responses. In a human cell line–based signaling assay, nicotinic acid stimulation led to pertussis toxin–sensitive lowering of cAMP, recruitment of \( \beta \)-arrestins to the cell membrane, an activating conformational change in \( \beta \)-arrestin, and \( \beta \)-arrestin–dependent signaling to ERK MAPK. In addition, we found that nicotinic acid promoted the binding of \( \beta \)-arrestin1 to activated cytosolic phospholipase \( A_2 \) as well as \( \beta \)-arrestin1–dependent activation of cytosolic phospholipase \( A_2 \) and release of arachidonate, the precursor of prostaglandin \( D_2 \) and the vasodilator responsible for the flushing response. Moreover, \( \beta \)-arrestin1–null mice displayed reduced cutaneous flushing in response to nicotinic acid, although the improvement in serum free fatty acid levels was similar to that observed in wild-type mice. These data suggest that the adverse side effect of cutaneous flushing is mediated by \( \beta \)-arrestin1, but lowering of serum free fatty acid levels is not. Furthermore, G protein–biased ligands that activate GPR109A in a \( \beta \)-arrestin–independent fashion may represent an improved therapeutic option for the treatment of dyslipidemia.

Introduction

GPCRs are by far the largest family of cellular receptors and the most important class of targets of therapeutic drugs. Recently, it has been discovered that these 7-transmembrane receptors (7TMRs) can evoke their cellular signals not only through hetero-trimeric G proteins, but also via \( \beta \)-arrestins, the multifunctional adaptor proteins originally discovered as desensitizers of GPCR function (1, 2). These discoveries have raised the issue of whether these 2 signaling mechanisms might contribute differently to the mediation of therapeutic versus adverse effects of drugs, and hence whether drugs that selectively activate or block one or the other of these pathways might offer therapeutic advantages (3).

Nicotinic acid, also known as niacin or vitamin B-3, has long been known to uniquely affect lipid profiles in humans. The pleiotropic effects of nicotinic acid therapy include the improvement of a number of cardiovascular risk factors. Specifically, nicotinic acid is one of the most effective drugs both for lowering triglycerides and for raising HDL. It has also been shown to lower VLDL and LDL levels (4). Nicotinic acid was the first lipid-modifying agent shown to have a beneficial effect on mortality (5). However, nicotinic acid therapy is associated with a very troublesome side effect: recipients experience a severe cutaneous vasodilation, or flushing, response, which often includes an intense burning and itching sensation (4). This occurs in approximately 80% of patients and frequently leads to discontinuation of the drug, thus dramatically limiting the efficacy of this potentially valuable therapeutic agent.

The lipid-modifying effects — especially those on triglycerides — and the cutaneous flushing elicited by nicotinic acid result from activation of GPR109A, a 7TMR GPCR, since both are lost in mice in which the receptor has been deleted by homologous recombination (6). Nicotinic acid–mediated stimulation of GPR109A receptors expressed on Langerhans cells in the skin leads to activation of cytosolic phospholipase \( A_2 \) (cPLA\(_2\)) and subsequent production and secretion of prostaglandin \( D_2 \) (PGD\(_2\)), which, through activation of its own 7TMR, leads to cutaneous flushing (7, 8). This cutaneous flushing response can be attenuated by inhibiting PGD\(_2\) synthesis with aspirin or by blocking PGD\(_2\) receptor with an antagonist (9–11). Neither of the above strategies is ideal, because they both involve administration of additional drugs. We sought a better understanding of the molecular signaling mechanisms responsible for the nicotinic acid–induced cutaneous flushing response with respect to G protein and \( \beta \)-arrestin involvement in the hopes that this might lead to alternative treatments with an improved side effect profile.

There are several different mechanisms by which \( \beta \)-arrestins mediate and modulate signaling of GPCRs. First, \( \beta \)-arrestins can inhibit G protein signaling. For instance, GPCR kinases (GRKs) rapidly phosphorylate agonist-activated 7TMRs. Phosphorylation of 7TMRs facilitates binding of \( \beta \)-arrestin, which effectively reduces these signals. In addition, \( \beta \)-arrestin bound to phosphorylated 7TMRs acts as an adaptor protein for the endocytic machinery by recruiting AP2 and clathrin (12). These events facilitate endocytosis via clathrin-coated pits and recycling of desensitized 7TMRs (13). Second, \( \beta \)-arrestins can mediate 7TMR signals independent of, or in concert with, G protein signaling. For example, \( \beta \)-arrestins and GRKs transport intracellular signal-
decreases cAMP, and this response is sensitive to pertussis toxin (19, 20). Stimulation of the receptor
β-arrestin1–mYFP or β-arrestin2–mYFP. In the absence of nicotinic acid, both β-arrestin1 and β-arrestin2 were localized primarily in the cytoplasm (Figure 1B), with a small amount of β-arrestin2 at the cell membrane. Stimulation with nicotinic acid resulted in robust recruitment of either β-arrestin–mYFP isoform to the cell membrane and a qualitative decrease in cytoplasmic fluorescence (Figure 1B).

To determine whether β-arrestins are expressed in cells mediating the physiologic response of GPR109A and whether β-arrestin recruitment occurs in response to activation of endogenous receptor, we examined β-arrestin expression in differentiated 3T3-L1 adipocytes, differentiated THP-1 macrophages, and Langerhans cells and also measured β-arrestin1 membrane recruitment in Langerhans cells. All 3 cell types expressed both β-arrestin1 and β-arrestin2 (Figure 2A). In the absence of nicotinic acid, β-arrestin1 remained primarily in the cytosol. Stimulation with nicotinic acid resulted in robust recruitment of β-arrestin1 to the membrane of Langerhans cells (Figure 2, B and C). Nicotinic acid stimulation also led to β-arrestin1 translocation in differentiated THP-1 macrophages (data not shown).

To further characterize the functional interaction of β-arrestin with GPR109A upon nicotinic acid stimulation, we used a recently described intramolecular bioluminescence resonance energy transfer–based (BRET-based) biosensor that detects conformational changes in β-arrestin2 associated with binding an activated 7TMR (21). This biosensor contains bioluminescent Renilla luciferase (Luc) and YFP fused at the N and C termini, respectively, of β-arrestin2 (referred to herein as Luc–β-arrestin–YFP). Upon recruitment to the receptor, β-arrestin undergoes receptor activation–dependent conformational changes that have been shown to alter the distance and/or orientation of Luc and YFP relative to each other, resulting in an increase in intramolecular BRET efficiency (21). Thus, the Luc–β-arrestin–YFP biosensor can be used as a reporter for receptor activation as well as for β-arrestin recruitment to the membrane. GPR109A clearly leads to activation of β-arrestin–dependent signaling; this pathway mediated the adverse side effect of cutaneous flushing, but not the beneficial antilipolytic effect of nicotinic acid.

Results
GPR109A has previously been shown to couple to the heterotrimeric G proteins G13/Gq, (19, 20). Stimulation of the receptor decreases cAMP, and this response is sensitive to pertussis toxin (14). To investigate the cellular signaling properties of GPR109A, we established GPR109A-expressing stable HEK-293 cell lines. Based on radioligand binding with nicotinic acid, these cells expressed the receptor at 1,300 fmol/mg total membrane protein (data not shown). After stimulation with nicotinic acid, cAMP decreased, and, as previously reported by others (14), this response was sensitive to pertussis toxin (Figure 1A).

Next we examined whether nicotinic acid–mediated stimulation promotes β-arrestin recruitment to the GPR109A receptor, and whether β-arrestins play a role in GPR109A-mediated signaling. GPR109A-expressing stable cells were transfected with either monomeric yellow fluorescent protein–tagged (mYFP-tagged) β-arrestin1 (referred to herein as β-arrestin1–mYFP) or β-arrestin2–mYFP. In the absence of nicotinic acid, both β-arrestin1 and β-arrestin2 were localized primarily in the cytoplasm (Figure 2A), with a small amount of β-arrestin2 at the cell membrane. Stimulation with nicotinic acid resulted in robust recruitment of either β-arrestin–mYFP isoform to the cell membrane and a qualitative decrease in cytoplasmic fluorescence (Figure 1B).
A receptor. Stimulation of HEK-293 cells coexpressing GPR109A and the Luc–β-arrestin–YFP biosensor by nicotinic acid led to an increase in intramolecular BRET ratio in a dose-dependent manner (Figure 3A). A 50% effective concentration (EC_{50}) of 1.45 ± 0.3 × 10^{-8} M was observed for the conformational change in β-arrestin, which corresponds well with the previously reported K_{B} of the GPR109A receptor (14) and with the observed IC_{50} for cAMP production (Figure 1). We also monitored real-time changes in intramolecular BRET ratio upon stimulation of cells with nicotinic acid. We observed a time-dependent conformational change in β-arrestin, with a t_{1/2} of maximal BRET increase of 53 ± 5 seconds (Figure 3B). This time course of conformational change in β-arrestin agrees well with that reported for other class A receptors using this biosensor (21).

Both β-arrestins and G proteins can mediate phosphorylation of ERK after agonist stimulation of G_{i}/G_{o}-coupled receptors, such as CCR7 and CXCR4 (22, 23). We examined whether G_{i}/G_{o} proteins mediated GPR109A-stimulated ERK activation in our stable cell lines in the presence or absence of pertussis toxin. Nicotinic acid did not activate ERK in control HEK-293 cells, which lack GPR109A (data not shown). Phosphorylation of ERK increased after agonist activation with nicotinic acid in GPR109A-expressing cells, and this response was all but eliminated by pertussis toxin, indicating involvement of G_{i}/G_{o} proteins in this response (Figure 4A and ref. 14). Next we examined whether the β-arrestins were also involved in GPR109A-stimulated ERK activation using siRNA targeting either β-arrestin1 or β-arrestin2. Following agonist stimulation, ERK was phosphorylated in control siRNA–transfected cells. In contrast, the response was largely abrogated in cells deplet-

Figure 2
Adipocytes, macrophages, and Langerhans cells express β-arrestins, and β-arrestin1 is recruited to the cell membrane with stimulation of GPR109A in Langerhans cells. (A) Cells lysates from differentiated 3T3-L1 adipocytes, differentiated THP-1 macrophages, and Langerhans cells (LHC) expressed both β-arrestin1 (Barr1) and β-arrestin2 (Barr2). (B) After stimulation with 10 μM nicotinic acid for 10 minutes, Langerhans cells were harvested, and membranes were separated from the cytosol, as demonstrated by presence of tubulin only in the cytosolic fractions. Increased β-arrestin1 was detected in the membranes after nicotinic acid stimulation, in contrast to control-treated samples. (C) Recruitment of β-arrestin1 to the membrane after nicotinic acid stimulation. *P = 0.0014 versus nicotinic acid. Data are mean ± SEM of 3 independent experiments.
acid, and this response was inhibited by depletion of β-arrestin1 with siRNA (Figure 6, B and D). To determine whether ERK or G<sub>i</sub>/G<sub>o</sub> proteins are involved in nicotinic acid–stimulated phosphorylation of cPLA<sub>2</sub>, we also measured this response after pretreatment of cells with either pertussis toxin or the ERK inhibitor PD98059. Both treatments substantially increased the basal level of phosphorylated cPLA<sub>2</sub>, and there was no further stimulation by nicotinic acid (Figure 6, B and D). However, because of this large increase in basal cPLA<sub>2</sub> activation after these treatments, it is unclear whether they also actually block nicotinic acid stimulation. Thus, we cannot firmly conclude that G<sub>i</sub>/G<sub>o</sub> or ERK are involved in this response. The interaction with cPLA<sub>2</sub> was specific for β-arrestin1, and activation of cPLA<sub>2</sub> required β-arrestin1. These data suggest that β-arrestin1 might be required for nicotinic acid–induced cutaneous flushing; moreover, β-arrestins and G proteins may contribute differentially to the therapeutic effects of nicotinic acid on lipids and on the undesired effect of cutaneous flushing.

Nicotinic acid has been shown to decrease serum FFAs and increase cutaneous blood flow in humans and in mice (4). Both of these responses require GPR109A, and the decrease in FFAs has also been

Figure 4
Nicotinic acid–stimulated phosphorylation of ERK. (A) GPR109A-expressing HEK-293 cells were stimulated with 200 μM nicotinic acid, and cell lysates were analyzed for phosphorylated ERK (pERK) at varying times. Agonist stimulated activation of ERK in the presence or absence of pertussis toxin. tERK, total ERK. *P = 0.027 versus control. (B) Expression of β-arrestin decreased after siRNA treatment. (C) Agonist stimulated ERK activation in the presence of control, β-arrestin1, β-arrestin2, or β-arrestin1 and β-arrestin2 siRNA. Graph shows phosphorylation of ERK 10 minutes after stimulation. **P < 0.05 versus control. Data are mean ± SEM of 3–6 independent experiments.

Figure 5
Nicotinic acid–induced binding of β-arrestin to cPLA<sub>2</sub> and phosphorylated cPLA<sub>2</sub>. (A and B) GPR109A-expressing HEK-293 cells were stimulated with 10 μM nicotinic acid or control for 10 minutes. Nicotinic acid stimulation increased binding of β-arrestin to cPLA<sub>2</sub> (A) and phosphorylated cPLA<sub>2</sub> (p-cPLA<sub>2</sub>) (B). Arrow indicates phosphorylated cPLA<sub>2</sub> band. Equivalent amounts of cPLA<sub>2</sub> were present in each whole cell lysate (WCL). Equal amounts of β-arrestin were immunoprecipitated in control and nicotinic acid–treated samples. Moreover, β-arrestin was not immunoprecipitated with preimmune serum (not shown). (C and D) Binding of β-arrestin to cPLA<sub>2</sub> (C) and phosphorylated cPLA<sub>2</sub> (D). *P = 0.0075, **P = 0.015 versus control. Data are mean ± SEM of 5 independent experiments.
shown to require G\textsubscript{i}/G\textsubscript{o} proteins (26). This nicotinic acid–induced decrease in serum FFAs has been used as a surrogate for its lipid-lowering effects. We studied the effect of nicotinic acid on serum FFAs in wild-type C57BL/6, β-arrestin1–deficient, and β-arrestin2–deficient mice. Injection of nicotinic acid i.p. to all 3 genotypes produced significant and essentially identical decreases in FFAs (Figure 7). Statistical analysis by 2-way ANOVA comparing the interaction of dose indicated $P < 0.0001$, and comparing for genotype indicated $P = 0.92$ (wild-type versus β-arrestin1) and $P = 0.94$ (wild-type versus β-arrestin2). Thus, neither β-arrestin1 nor β-arrestin2 was required for nicotinic acid–induced changes in serum FFAs.

Additionally, we examined changes in cutaneous flushing and eicosanoid release after administration of nicotinic acid by measuring perfusion of the ventral mouse ear using laser Doppler perfusion imaging in vivo and determining cPLA\textsubscript{2} activity in mouse macrophages ex vivo. Injection of nicotinic acid i.p. to all 3 genotypes produced significant and essentially identical decreases in FFAs (Figure 7). Statistical analysis by 2-way ANOVA comparing the interaction of dose indicated $P < 0.0001$, and comparing for genotype indicated $P = 0.92$ (wild-type versus β-arrestin1) and $P = 0.94$ (wild-type versus β-arrestin2). Thus, neither β-arrestin1 nor β-arrestin2 was required for nicotinic acid–induced changes in serum FFAs.

Figure 6
Role of β-arrestin1 in binding and activation of cPLA\textsubscript{2}. (A) GPR109A-expressing HEK-293 cells were transfected with FLAG–β-arrestin1 or FLAG–β-arrestin2. Nicotinic acid stimulation increased binding of cPLA\textsubscript{2} to FLAG–β-arrestin1, but not FLAG–β-arrestin2. (B) Equivalent amounts of cPLA\textsubscript{2} and FLAG–β-arrestins were present in each whole cell lysate. Equal amounts of FLAG–β-arrestin were immunoprecipitated in control and nicotinic acid–treated samples. GPR109A-expressing HEK-293 cells were stimulated with 200 μM nicotinic acid, and cell lysates were analyzed for phosphorylated cPLA\textsubscript{2} at varying times. Agonist-stimulated activation of cPLA\textsubscript{2} in the presence of control siRNA, β-arrestin1 siRNA, or control siRNA plus either pertussis toxin or PD98059 (PD). (C) Binding of FLAG–β-arrestin to cPLA\textsubscript{2}. *$P = 0.0004$ versus respective control. (D) Activation or phosphorylation of cPLA\textsubscript{2} in siRNA-treated cells. **$P = 0.0085$ versus respective 10-minute value; ***$P = 0.0047$ versus respective 0-minute value. Data are mean ± SEM of 3 independent experiments.
of cPLA2 activity, increased after stimulation with nicotinic acid in wild-type and β-arrestin2–deficient macrophages, and this response was significantly reduced in β-arrestin1–deficient cells (Figure 8E). We observed a nonsignificant trend toward diminished cutaneous flushing in β-arrestin2–deficient mice; hence, we speculate that β-arrestin2 could also play some role in this response and may account for part of the residual eicosanoid production in β-arrestin1–deficient macrophages. While we cannot exclude the possibility that defective nicotinic acid–induced cutaneous flushing in β-arrestin1–deficient mice involves additional mechanisms, defective cPLA2 activity in immune cells markedly limited cutaneous flushing in these animals. Taken together, these findings demonstrate that the adverse side effect of cutaneous flushing associated with the administration of nicotinic acid is mediated by β-arrestin1. In contrast, the effects on serum FFAs are mediated by β-arrestin–independent — G\textsubscript{i}/G\textsubscript{o} protein dependent — mechanisms, which have previously been shown to be pertussis toxin sensitive (26).

Recently developed GPR109A agonists, such as MK-0354, decrease serum FFAs, but do not inducing cutaneous flushing (19, 27, 28). We wondered whether biased signaling toward G\textsubscript{i}/G\textsubscript{o} proteins might be the mechanism for such biased or selective pharmacology. To test this hypothesis, we measured G protein signaling and β-arrestin recruitment after agonist activation of GPR109A-expressing HEK-293 cells using the agonist MK-0354. As previously demonstrated (28), stimulation of the receptor with MK-0354 decreased cAMP, and this response was sensitive to pertussis toxin (Figure 9A). However, stimulation with MK-0354 failed to induce a conformational change in the Lue-β-arrestin1–YFP biosensor as measured by BRET (Figure 9B). Moreover, MK-0354 failed to induce recruitment of β-arrestin1–mYFP to the cell membrane and inhibited nicotinic acid–induced recruitment (Figure 9C). Hence, a nonflushing agonist of GPR109A activated G protein signaling, but failed to stimulate recruitment of β-arrestin, perhaps explaining its selective pharmacology. These findings have 2 possible explanations: that MK-0354 is a G protein–biased agonist, or that MK-0354 is a weak partial agonist. Both of these explanations may be consistent with this compound’s reported efficacy for FFA lowering in the absence of cutaneous flushing. This selective effect could be achieved by either a biased ligand that engages G protein coupling but not β-arrestin coupling, or by a partial agonist that weakly engages both G protein and β-arrestin coupling and gains selectivity for the G protein response through downstream amplification, which is commonly seen for G protein–coupled responses.

**Discussion**

Nicotinic acid inhibits lipolysis in adipocytes, decreasing serum FFAs and triglycerides, and this effect is mediated by GPR109A (14). In addition to the triglyceride-lowering effect, nicotinic acid also improves a number of other cardiovascular risk factors. Specifically, nicotinic acid is the most effective HDL-raising therapy currently known, and has also been shown to lower both VLDL and LDL (4). While the FFA- and triglyceride-lowering effects of nicotinic acid are clearly mediated by GPR109A, it is not yet known whether its beneficial effects on HDL, VLDL, and LDL are also mediated by this receptor (29). GPR109A receptors are found primarily in adipose tissue, spleen, adrenal glands, and lungs, and are all but absent from the liver and intestines, which are the main sites of HDL synthesis and metabolism (14, 30). Thus, there may be additional mechanisms of action for the beneficial effects of nicotinic acid on lipoprotein profiles, mediated through sites other than GPR109A. For example, nicotinic acid at high concentrations has been shown to directly inhibit hepatic diacylglycerol acetyltransferase (31, 32), thus inhibiting hepatic triglyceride synthesis, which increases apoB degradation and consequently decreases VLDL and LDL production and secretion. Nicotinic acid has also been shown, via an as-yet-unidentified mechanism, to inhibit the uptake and removal of HDL by the liver, resulting in increased circulating HDL levels (33). Thus, multiple sites of action may be involved in the pleiotropic actions of nicotinic acid on lipoproteins beyond the clear effect on FFAs and triglycerides mediated through GPR109A. However, GPR109A clearly mediates the prominent side effect of cutaneous flushing (6), and does so in a β-arrestin1–dependent fashion. This is in contrast to the desired therapeutic effects on FFAs that are not mediated via β-arrestins.

We have previously shown for the AT1 angiotensin receptor, parathyroid hormone receptor, and β\textsubscript{2}-adrenergic receptor that it is possible to selectively induce either G protein– or β-arrestin–biased signaling with specific ligands (34–37). Such molecules entrain subsets of receptor signaling pathways without activating all of a receptor’s possible downstream effectors. This idea of biased ligands departs from the traditional view of receptor ligands as full agonists, partial agonists, inverse agonists, or antagonists and opens up a much more nuanced framework in which receptor ligands might act independently to activate either G proteins or β-arrestins (38). As potential therapeutic agents, such ligands could specifically target therapeutic effectors while avoiding those signaling pathways associated with particular side effects. Indeed, recent studies using novel GPR109A agonists that decrease serum FFAs in mice and humans without inducing cutaneous flushing demonstrated divergent signaling pathways downstream of GPR109A activation (19, 27, 28). Specifically, compounds such as MK-0354 activate G proteins, but fail to induce ERK activation and internalization of the receptor. Our observations suggest that MK-0354 preferentially activates G proteins over β-arrestins, further supporting the notion that it is possible to specifically target

**Figure 7**

Nicotinic acid induces antilipolysis in wild-type and β-arrestin–deficient mice. Nicotinic acid decreased FFA levels in wild-type C57BL/6 mice as well as mice deficient in β-arrestin1 or β-arrestin2. Nonesternivated FFAs were measured in mice given i.p. injections of either vehicle alone or nicotinic acid at a dose of 10, 50, or 100 mg/kg. FFA levels are expressed as a percent of vehicle-treated control animals for each genotype. *P < 0.0001 comparing the interaction of dose. The change in FFAs after agonist stimulation was not significantly different between wild-type, β-arrestin1–deficient, and β-arrestin2–deficient mice. Data are mean ± SEM in control or nicotinic acid–treated animals (n = 4–10 per condition).
the beneficial effects of GPR109A signaling while avoiding signaling pathways that mediate cutaneous flushing.

While most patients taking nicotinic acid experience cutaneous flushing, some are able to tolerate this side effect, mostly because tolerance to cutaneous flushing sometimes occurs with prolonged use of the medication. The mechanism of tolerance to nicotinic acid–induced cutaneous flushing is not understood, and such information may also lead to improvements in nicotinic acid–based therapies. The identification of GPR109A as a receptor for nicotinic acid (6), coupled with the finding that β-arrestin proteins are recruited after activation of this receptor, leads us to speculate that β-arrestins may also internalize GPR109A and desensitize GPR109A-mediated signaling. Hence, these proteins may also play a role in tolerance to nicotinic acid–induced cutaneous flushing. However, since β-arrestin–mediated receptor internalization would also be predicted to desensitize G protein signaling, and tolerance to the beneficial effects of nicotinic acid on serum lipids does not occur, the role of β-arrestins in desensitization of GPR109A-mediated signaling is likely to be complicated.

In summary, the adverse side effect of cutaneous flushing associated with nicotinic acid was mediated by β-arrestin1, while the effects on lowering serum FFAs were not. Thus, agents that possess the FFA- and triglyceride-altering attributes of nicotinic acid but do not activate β-arrestin recruitment to GPR109A can be predicted to lack the side effect of cutaneous flushing. Such biased ligands would provide a significant therapeutic advantage over currently available medications used to treat hypertriglyceridemia and potentially other dyslipidemias. Moreover, screening for GPR109A agonists that stimulate activation of G proteins but not β-arrestin1 provides a strategy for their identification. These findings provide a striking example of how desired therapeutic and unwanted side effects of GPCR-targeted drugs can be dissociated with respect to molecular signaling pathways through G proteins and β-arrestins.
Methods

Materials. Nicotinic acid, PGD₂, and (2-hydroxymethyl)-β-cyclodextrin were obtained from Sigma-Aldrich. MK-0354 was a gift from J. Richman (Arena Pharmaceuticals Inc., San Diego, California, USA). Pertussis toxin and the ERK inhibitor PD98059 were obtained from Calbiochem. Nicotinic acid [5,6-3H] was obtained from American Radiolabeled Chemicals. Arachidonic acid [5,6,8,9,12,14,15-3H(N)] was obtained from PerkinElmer. Coelenterazine h was purchased from Promega, and 96-well microplates for the BRET assay were purchased from Corning Inc.

Plasmids. We generated β-arrestin1–mYFP, β-arrestin2–mYFP, FLAG–β-arrestin1, and FLAG–β-arrestin2 in our laboratory (39). FLAG-GPR109A/pcDNA3.1 was a gift from S. Offermanns (University of Heidelberg, Heidelberg, Germany). The Luc–β-arrestin–YFP construct was provided by M. Bouvier (Université de Montréal, Montréal, Quebec, Canada).

Cell culture. We maintained 3T3-L1 cells in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (Sigma-Aldrich), and differentiated the cells by allowing them to reach confluence. THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, 1 mM sodium pyruvate, 10 mM HEPES, 4.5 g/l glucose, 1.5 g/l bicarbonate, and 0.05 mM 2-mercaptoethanol and were differentiated as previously described (40). Langerhans cells were purchased and maintained according to the manufacturer’s instructions (MatTek Corp.). HEK-293 cells were maintained in modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Sigma-Aldrich). Cells were transfected with FuGENE 6 (Roche Applied Science). All transfections used 3 μg plasmid in a 10-cm tissue culture plate. Cells expressing GPR109A alone were selected with 400 μg/ml G418 (Sigma-Aldrich), and colonies of stable transfectants were isolated. Cells expressing GPR109A in combination with the ICUE2 biosensor were selected with 400 μg/ml G418 and 300 μg/ml Zeocin (Invitrogen).

ICUE cAMP assay. HEK-293 cells stably overexpressing both FLAG-GPR109A and the cAMP biosensor ICUE2 were stimulated with nicotinic acid or MK-0354 for 3 minutes, followed by stimulation with 10 μM forskolin for 4 minutes. Intracellular cAMP concentrations were measured as a fluorescence resonance energy transfer (FRET) ratio as follows: cyan fluorescent protein (CFP) intensity (438/32 nm emission bandpass filters; Semrock) relative to FRET intensity (542/27 emission filter; refs. 41, 42). Experiments were performed on a NOVOstar plate reader (BMG Labtech).

β-Arrestin translocation assays. HEK-293 cells stably expressing FLAG-GPR109A were transiently transfected with β-arrestin1–mYFP or β-arrestin2–mYFP using FuGENE 6 (Roche Applied Science). Cells were treated with nicotinic acid or MK-0354, and images were taken at 5-minute intervals after stimulation. Differentiated THP-1 cells were serum starved for 6 hours and subsequently stimulated with 200 μM nicotinic acid for 10 minutes. The plates were transferred on ice, and cells were washed twice with ice-cold PBS and then scraped in PBS containing complete protease inhibitor cocktail (Roche Applied Sciences). Cells were lysed by brief sonication and then centrifuged at 3,000 × g for 5 minutes to separate the membrane fraction (in the pellet) and cytosolic fractions. Subsequently, the supernatant was centrifuged at 21,000 × g for 30 minutes to separate the membrane fraction (in the pellet) and cytosolic fraction (in the supernatant). The membrane pellet was resuspended in PBS, proteins in the membrane and the cytosolic fractions were measured by Bradford assay, and β-arrestins were detected by Western blot analysis.

BRET assay. BRET assays were performed as described previously (21). Briefly, at 24 hours after transfection, HEK-293 cells coexpressing the receptor and the biosensor were distributed in fibronectin-coated 96-well microplates (white well, clear bottom). Before the assay, cells were washed twice with PBS, the transparent bottom of the plate was covered with a white back-tape adhesive, and cells were incubated with coelenterazine h (final concentration, 5 μM) for 10 minutes. Addition of coelenterazine h, a Renilla luciferase substrate, leads to emission of light upon oxidation of coelenterazine h to coelenteramide h, with a peak wavelength around 480 nm. This light energy is then transferred to YFP, provided that the YFP is within an appropriate distance (i.e., 10 nm) and/or orientation, and in turn results in energy emission with a peak wavelength around 530 nm. Subsequent to addition of coelenterazine h, the cells were stimulated with nicotinic acid or forskolin for 5 minutes. Intracellular cAMP concentrations were measured as a fluorescence resonance energy transfer (FRET) ratio as follows: cyan fluorescent protein (CFP) intensity (438/32 nm emission bandpass filters; Semrock) relative to FRET intensity (542/27 emission filter; refs. 41, 42). Experiments were performed on a NOVOstar plate reader (BMG Labtech).

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or MK-0354, and light emission was detected (460–500 nm for Luc and 510–550 nm for YFP) using a Multilabel Reader Mithras LB 940 (Berthold Technologies). The BRET signal was determined as the ratio of the light emitted by YFP and the light emitted by Luc. For dose response curves, different concentrations of ligands were used, and the BRET ratio was monitored at 10 minutes after ligand stimulation. For time kinetics, 10 μM nicotinic acid was added to the cells, and real-time change in BRET was monitored over 15 minutes. The values were corrected by subtracting the background BRET signals detected when Luc-β-arrestin was expressed alone.

**Immunoblotting and immunoprecipitation.** Phosphorylated ERK immunoblotting using the antibody anti-phospho-p44/p42 MAPK (diluted 1:2,000; Cell Signaling Technology) was carried out as previously described (37). Total ERK1/2 was detected with anti-MAPK1/2 (diluted 1:3,000; Upstate Biotechnology). Detection of β-arrestin1 and β-arrestin2 was performed by using rabbit polyclonal antibodies (A1CT and A2CT, respectively) that were generated in our laboratory (43). Anti-rabbit and anti-mouse secondary antibodies for Western blots were obtained from Amersham Biosciences. For immunoprecipitation, cells were treated with serum-free media with and without nicotinic acid. Cells were washed once with PBS at 4°C, harvested by gentle scraping, pelleted, and resuspended in glycerol lysis buffer including protease and phosphatase inhibitors. Lysates were normalized for equal protein concentrations and immunoprecipitated with A1CT or conjugated M2-beads (Sigma-Aldrich) (43). Immunoprecipitation reactions were incubated at 4°C for 3 hours, washed 3 times with glycerol lysis buffer, and resuspended in SDS running buffer. Samples were subjected to SDS-PAGE analysis and Western blotting with cPLA2 antibody (diluted 1:1,000; Cell Signaling Technology), phosphorylated cPLA2 antibody (diluted 1:1,000; Cell Signaling Technology), M2 antibody (diluted 1:2,000; Sigma-Aldrich), and β-arrestin antibody (diluted 1:1,000; BD Biosciences).

Silencing of gene expression with siRNA. siRNA gene silencing was carried out with previously described siRNAs and methods (44). Protein silencing and eicosanoid analysis and Western blotting with cPLA2 antibody (diluted 1:1,000; Cell Signaling Technology), phosphorylated cPLA2 antibody (diluted 1:1,000; Cell Signaling Technology), M2 antibody (diluted 1:2,000; Sigma-Aldrich), and β-arrestin antibody (diluted 1:1,000; BD Biosciences).

**Animal use and protocols.** All animal studies were reviewed and approved by the Duke University Internal Animal Care and Use Committee. Congenic C57BL/6 wild-type mice, β-arrestin1-depleted mice, and β-arrestin2-depleted mice were developed and maintained as previously described (17). Briefly, congenic C57BL/6 wild-type mice, β-arrestin1-deficient, or β-arrestin2-deficient animals were bred, and progeny genotypes were confirmed by PCR and Southern blots. Age- and weight-matched male mice over 12 weeks of age were used in all experiments. Nicotinic acid was resuspended in 5% (2-hydroxypropyl)β-cyclodextrin in PBS, and the pH was adjusted to 7.4. For FFA assays, mice were food deprived for 8 hours, then treated with 0, 10, 50, or 100 mg/kg nicotinic acid administered by i.p. injection. The animals were euthanized 30 minutes later. Serum was collected and stored at –80°C. Nonesterified FFAs were measured using a Hitachi 911 clinical autoanalyzer, with standards and reagents from Wako USA as previously described (45). For mouse cutaneous flushing assays, mice were anesthetized with nembutal (80 mg/kg) via i.p. injection. After 10 minutes, the mice were placed under an LDPI laser Doppler (PeriScan PIM II, Perimed). The right eye was everted to expose the anterior/ventral surface. The laser Doppler was focused on the central portion of the ventral ear. Data were collected using the repeated data collection mode with a 5-mm × 5-mm image size, a 1-second delay, and high-resolution scan. After a 5-minute baseline scan was obtained, 100 mg/kg nicotinic acid was injected in the i.p. space. Readings were continually recorded for 30 minutes. As a control, each animal was subsequently treated with 4 mg/kg PGZ2 dissolved in PBS. For eicosanoid release assays, thioglycollate-elicited peritoneal macrophages were collected as previously described (46, 47). Cells were pretreated with 0.1 μCi/ml H3-arachidonic acid for 24 hours, then rinsed 5 times to remove unincorporated H3-arachidonic acid (48). Macrophages (1 × 106 cells/well) were stimulated for 10 minutes with 200 μM nicotinic acid, and radioactivity released into the media was measured using a Packard 2700 TR liquid scintillation counter.

**Statistics.** Significance of differences was determined by 2-way ANOVA with post-hoc Bonferroni tests or 2-tailed Student’s paired t tests, using Prism software (version 4; GraphPad). A P value less than 0.05 was considered statistically significant.

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