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Research Article

Cardiology

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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/G_o proteins, here we dissected the roles of G proteins and the adaptor proteins, β -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 β -arrestins to the cell membrane, an activating conformational change in β -arrestin, and β -arrestin–dependent signaling to ERK MAPK. In addition, we found that nicotinic acid promoted the binding of β -arrestin1 to activated cytosolic phospholipase A₂ as well as β -arrestin1–dependent activation of cytosolic phospholipase A₂ and release of arachidonate, the precursor of prostaglandin D₂ and the vasodilator responsible for the flushing response. Moreover, β -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 β -arrestin1, but lowering of serum free fatty acid levels is not. Furthermore, G protein–biased ligands that activate GPR109A in a β -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 heterotrimeric G proteins, but also via β -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 differentially 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.

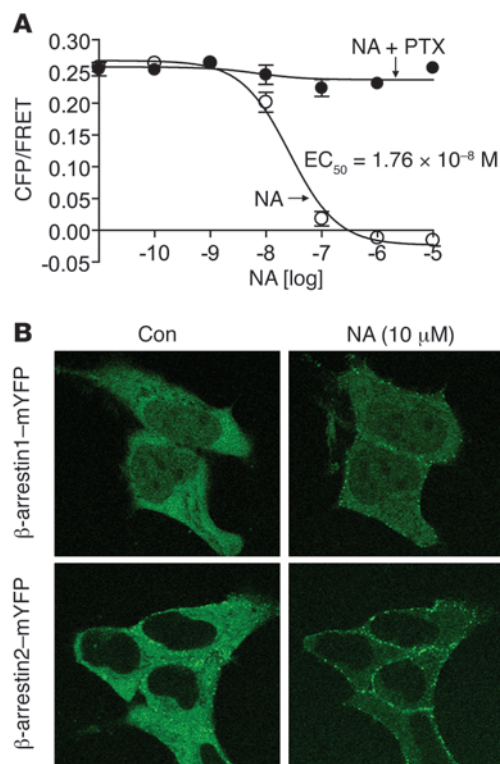
The lipid-modifying effects — especially those on triglycerides — and the cutaneous flushing elicited by nicotinic acid result from activation of GPR109A, a 7TMR GPCR, since both are lost in mice in which the receptor has been deleted by homologous recombination (6). Nicotinic acid–mediated stimulation of GPR109A receptors expressed on Langerhans cells in the skin leads to activation of cytosolic phospholipase A₂ (cPLA₂) and subsequent production and secretion of prostaglandin D₂ (PGD₂), which, through activation of its own 7TMR, leads to cutaneous flushing (7, 8). This cutaneous flushing response can be attenuated by inhibiting PGD₂ synthesis with aspirin or by blocking PGD₂ 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 β -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 β -arrestins mediate and modulate signaling of GPCRs. First, β -arrestins can inhibit G protein signaling. For instance, GPCR kinases (GRKs) rapidly phosphorylate agonist-activated 7TMRs. Phosphorylation of 7TMRs facilitates binding of β -arrestin, which effectively reduces these signals. In addition, β -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, β -arrestins can mediate 7TMR signals independent of, or in concert with, G protein signaling. For example, β -arrestins and GRKs transport intracellular signal-

Conflict of interest: R.J. Lefkowitz is a scientific founder of Trevena, a company that is developing GPCR-targeted drugs.

Nonstandard abbreviations used: BRET, bioluminescence resonance energy transfer; cPLA₂, cytosolic phospholipase A₂; EC₅₀, 50% effective concentration; mYFP, monomeric YFP; 7TMR, 7-transmembrane receptor; YFP, yellow fluorescent protein.

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**Figure 1**

Nicotinic acid-induced decrease in cAMP, and increase in β -arrestin membrane recruitment, in GPR109A-expressing HEK-293 cells. Cells also expressing the ICUE2 biosensor (see Methods) were treated with forskolin and increasing concentrations of nicotinic acid (NA). **(A)** Nicotinic acid (open circles) decreased cAMP in a dose-dependent fashion, and this response was inhibited by pertussis toxin (PTX; filled circles). Data are mean \pm SEM of 3 independent experiments. CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer. **(B)** Cells were transfected with either β -arrestin1-mYFP or β -arrestin2-mYFP. Both β -arrestin isoforms resided in the cytosol prior to nicotinic acid stimulation (control; Con), and translocated to bind GPR109A in the membrane in response to treatment with 10 μ M nicotinic acid. No translocation was noted in cells lacking GPR109A (not shown). Images are representative of 4 independent experiments. Original magnification, $\times 100$.

ing complexes to agonist-activated 7TMRs, effectively redirecting or amplifying these signals (13). Additionally, G proteins and/or β -arrestins may transduce unique intracellular signals with distinct functional consequences. While nicotinic acid stimulation of GPR109A clearly leads to activation of G_i/G_o proteins (14), to our knowledge, no examination of the interaction of the receptor with β -arrestins has been previously reported.

Recently, it has been appreciated that these distinct signaling processes — G protein-dependent and β -arrestin-dependent — may differentially contribute to the desired therapeutic effects and the undesired side effects of modern pharmaceuticals. In this context, Bohn et al. have shown that there is little or no tolerance to the analgesic effects of morphine, as well as diminished morphine side effects, such as constipation and respiratory depression, in β -arrestin2-deficient mice compared with wild-type mice (15–18). These findings clearly demonstrate that one can differentiate between receptor-stimulated G protein-mediated effects (analgesia) and β -arrestin-mediated effects (tolerance, constipation, and respiratory depression). In view of these previous findings, we sought to examine whether β -arrestins mediate nicotinic acid-induced signaling from GPR109A, and whether they mediate either the beneficial effects of nicotinic acid on lipids, the significant side effect of cutaneous flushing, both, or neither. We found that nicotinic acid-induced stimulation of GPR109A led to β -arrestin-dependent signaling; this pathway mediated the adverse side effect of cutaneous flushing, but not the beneficial antilipolytic effect of nicotinic acid.

Results

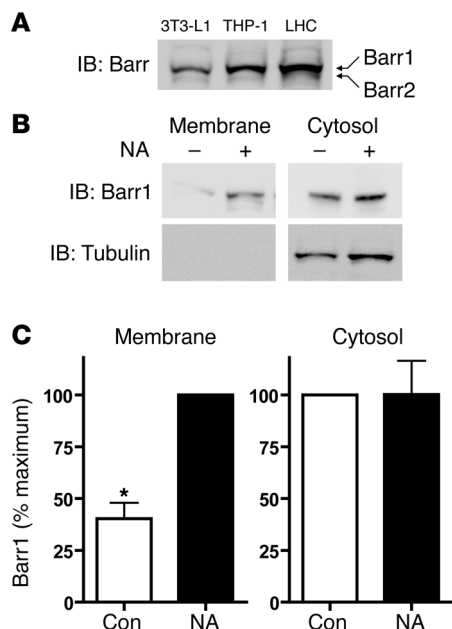
GPR109A has previously been shown to couple to the heterotrimeric G proteins G_i/G_o (19, 20). Stimulation of the receptor decreases cAMP, and this response is sensitive to pertussis toxin

(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 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 -arr-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 -arr-YFP biosensor can be used as a reporter for receptor activation as well as for β -arrestin recruitment to the



receptor. Stimulation of HEK-293 cells coexpressing GPR109A and the Luc-β-arr-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 $1.45 \pm 0.3 \times 10^{-8}$ M was observed for the conformational change in β-arrestin, which corresponds well with the previously reported K_d 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 depleted

Figure 3

Conformational change in β-arrestin2 upon activation of GPR109A. **(A)** Dose dependency of the conformational changes in β-arrestin2 upon stimulation of GPR109A by nicotinic acid. Filled squares, cells expressing Luc-β-arr-YFP and empty vector; open squares, cells expressing Luc-β-arr-YFP and GPR109A. **(B)** Kinetics of nicotinic acid-induced conformational change in β-arrestin. Data are mean \pm SEM of 4 independent experiments.

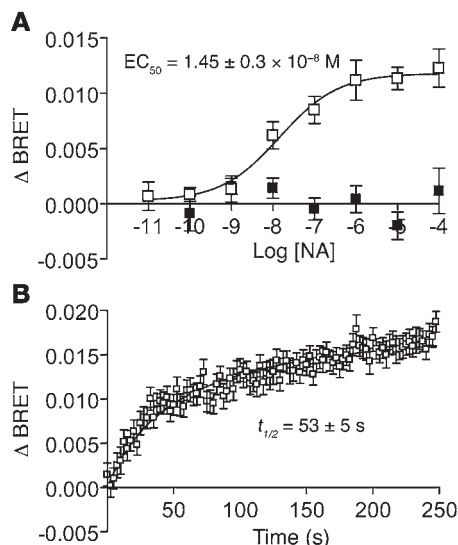
Figure 2

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)** Cell 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 \pm SEM of 3 independent experiments.

of β-arrestin1, β-arrestin2, or both ($P = 0.0023$, $P = 0.0042$, and $P = 0.0017$, respectively, versus control, paired 2-tailed Student's t test; Figure 4, B and C). Hence, nicotinic acid stimulation of ERK via GPR109A required both G_i/G_o proteins (pertussis toxin sensitive) and β-arrestins, consistent with prior findings for other G_i/G_o -coupled receptors, including CCR7 and CXCR4 (22, 23).

ERK phosphorylates and activates cPLA₂, a key enzyme in the production of PGD₂ via production of its precursor arachidonate (24). Because β-arrestin1 is known to interact with other phospholipases, such as phospholipase A₁, in an agonist-dependent manner (25), we examined whether β-arrestin interacts with cPLA₂ after stimulation of GPR109A. After stimulation with nicotinic acid, binding of β-arrestin to cPLA₂ and phosphorylated cPLA₂ (the activated form) increased compared with control-treated cells (Figure 5, A–D). To determine whether cPLA₂ interacts with β-arrestin1, β-arrestin2, or both, we transfected GPR109A-expressing cells with FLAG-tagged β-arrestin1 or β-arrestin2 and examined cPLA₂ binding to FLAG-β-arrestin. In control-treated cells, low levels of cPLA₂ were bound to both β-arrestin1 and β-arrestin2. After stimulation of GPR109A, binding of cPLA₂ to β-arrestin1 increased, and binding to β-arrestin2 was unchanged (Figure 6, A and C).

To investigate the role of β-arrestin1 in nicotinic acid-stimulated activation of cPLA₂, we measured phosphorylation of cPLA₂ after nicotinic acid stimulation in GPR109A-expressing cells. We observed an increase in phosphorylated cPLA₂ with nicotinic



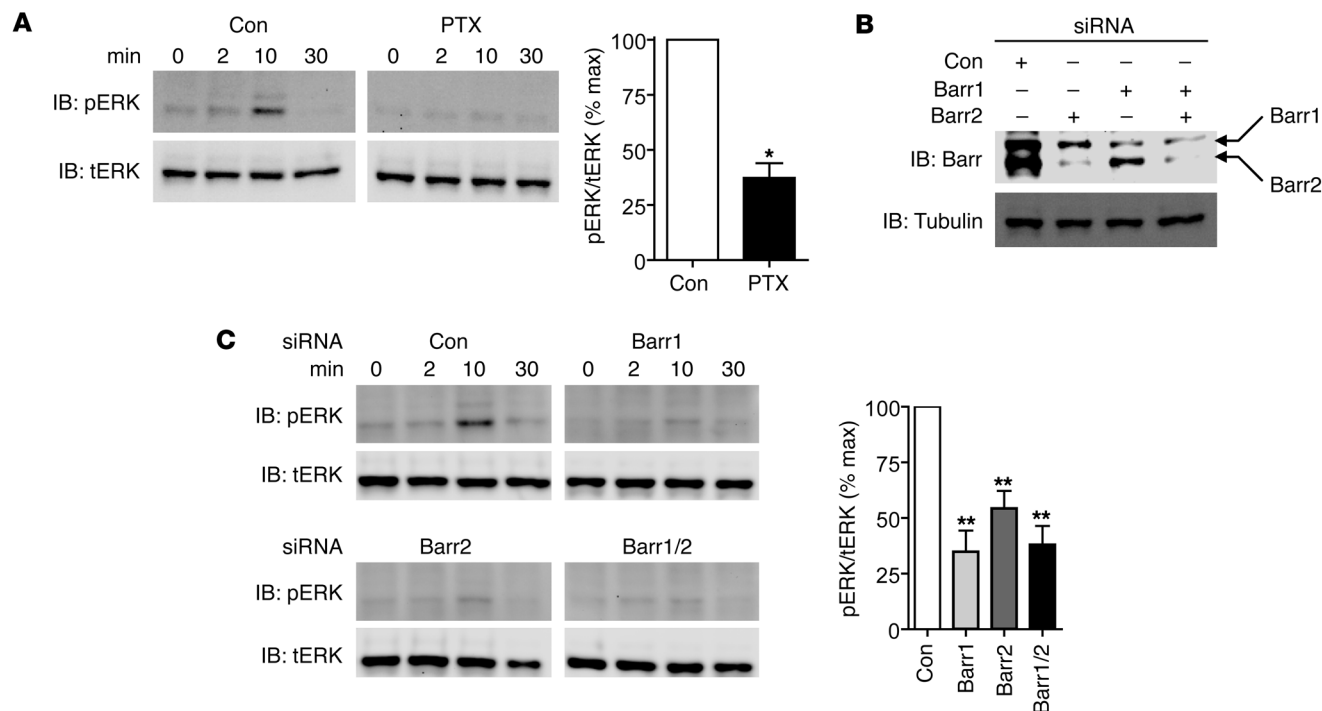


Figure 4

Nicotinic acid-stimulated phosphorylation of ERK. (A) GPR109A-expressing HEK-293 cells were stimulated with 200 μ M 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 \pm SEM of 3–6 independent experiments.

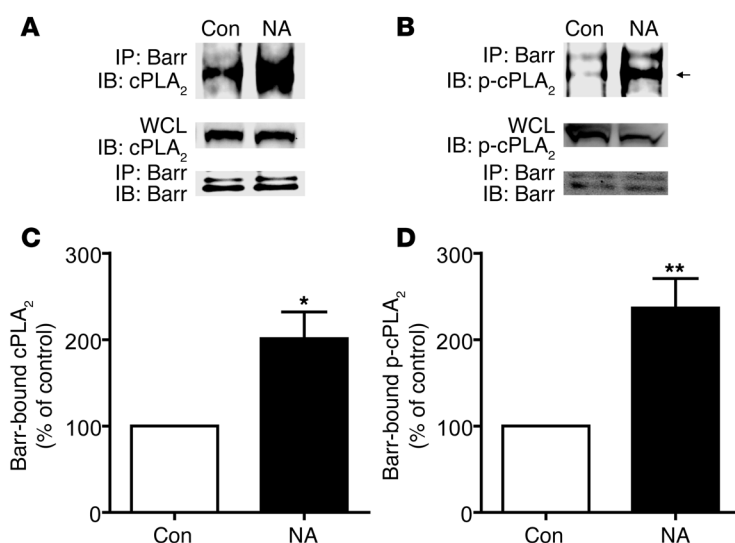
acid, and this response was inhibited by depletion of β -arrestin1 with siRNA (Figure 6, B and D). To determine whether ERK or G_i/G_o proteins are involved in nicotinic acid-stimulated phosphorylation of cPLA₂, we also measured this response after pretreatment of cells with either pertussis toxin or the ERK inhibitor PD98059. Both treatments substantially increased the basal level of phosphorylated cPLA₂, and there was no further stimulation by nicotinic acid (Figure 6, B and D). However, because of this large increase in basal cPLA₂ activation after these treatments, it is unclear whether they also actually block nicotinic acid stimulation. Thus, we cannot firmly conclude that G_i/G_o or ERK are involved in this response. The interaction with cPLA₂ was specific for β -arrestin1, and

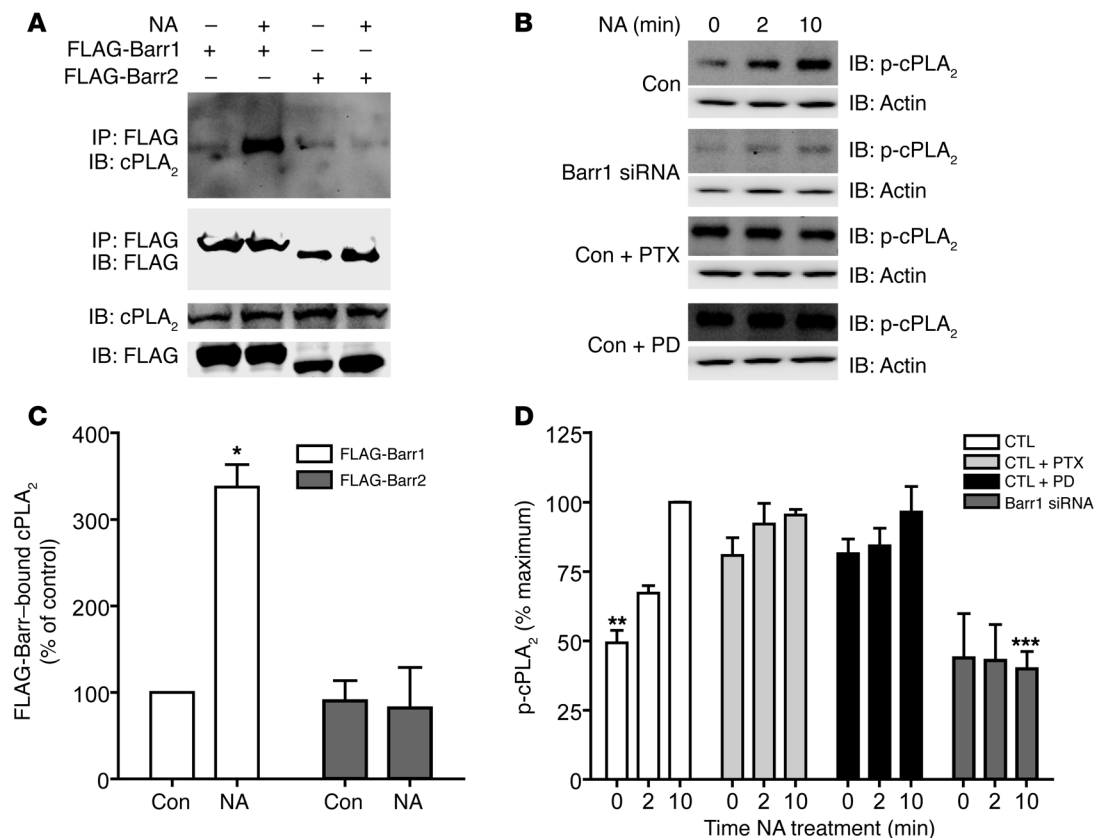
activation of cPLA₂ required β -arrestin1. These data suggest that β -arrestin1 might be required for nicotinic acid-induced cutaneous flushing; moreover, β -arrestins and G proteins may contribute differentially to the therapeutic effects of nicotinic acid on lipids and on the undesired effect of cutaneous 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 5

Nicotinic acid-induced binding of β -arrestin to cPLA₂ and phosphorylated cPLA₂. (A and B) GPR109A-expressing HEK-293 cells were stimulated with 10 μ M nicotinic acid or control for 10 minutes. Nicotinic acid stimulation increased binding of β -arrestin to cPLA₂ (A) and phosphorylated cPLA₂ (p-cPLA₂) (B). Arrow indicates phosphorylated cPLA₂ band. Equivalent amounts of cPLA₂ 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 cPLA₂ (C) and phosphorylated cPLA₂ (D). * $P = 0.0075$, ** $P = 0.015$ versus control. Data are mean \pm SEM of 5 independent experiments.



**Figure 6**

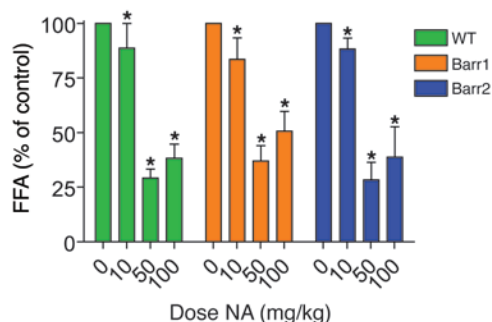
Role of β -arrestin1 in binding and activation of cPLA₂. (A) GPR109A-expressing HEK-293 cells were transfected with FLAG- β -arrestin1 or FLAG- β -arrestin2. Nicotinic acid stimulation increased binding of cPLA₂ to FLAG- β -arrestin1, but not FLAG- β -arrestin2. (B) Equivalent amounts of cPLA₂ and FLAG- 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 μ M nicotinic acid, and cell lysates were analyzed for phosphorylated cPLA₂ at varying times. Agonist-stimulated activation of cPLA₂ 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 cPLA₂. * $P = 0.0004$ versus respective control. (D) Activation or phosphorylation of cPLA₂ in siRNA-treated cells. ** $P = 0.0085$ versus respective 10-minute value; *** $P = 0.0047$ versus respective 0-minute value. Data are mean \pm SEM of 3 independent experiments.

shown to require G_i/G_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 cPLA₂ activity in mouse macrophages ex vivo. Injection of nicotinic acid i.p. led to a dramatic increase in perfusion of the ventral ear in both wild-type and β -arrestin2-deficient mice (Figure 8, A–C). Mice deficient in β -arrestin2 showed a trend toward decreased cutaneous flushing that was not statistically significant by 2-way ANOVA, even at the higher 200-mg/kg dose of nicotinic acid (data not shown). Sur-

prisingly, the nicotinic acid-stimulated increase in perfusion was dramatically decreased in the β -arrestin1-deficient mice (Figure 8, A–C). Analysis by 2-way ANOVA comparing wild-type with β -arrestin1-deficient mice indicated $P = 0.0001$, and wild-type compared with β -arrestin2-deficient mice indicated $P = 0.39$. As a control, we measured perfusion after injection of PGD₂, the downstream mediator of nicotinic acid-induced vasodilation. Cutaneous flushing increased with PGD₂ in all 3 genotypes, and the response among wild-type, β -arrestin1-deficient, and β -arrestin2-deficient animals was comparable (Figure 8D). These data clearly demonstrate that β -arrestin1 participates in mediating nicotinic acid-induced cutaneous flushing and that the defect in cutaneous flushing occurs upstream of the actions of prostaglandin on cutaneous blood vessels (i.e., upstream of prostaglandin release).

To further investigate the mechanism by which β -arrestin1 mediates the cutaneous flushing response, we harvested peritoneal macrophages from wild-type, β -arrestin1-deficient, and β -arrestin2-deficient mice and measured cPLA₂ activity in these cells after stimulation of GPR109A. Macrophages were pretreated with H³-arachidonic acid for 24 hours to allow incorporation into membrane lipids. Release of radiolabeled eicosanoids, a measure

**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. Nonesterified 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 nicotinic acid stimulation was not significantly different between wild-type, β -arrestin1-deficient, and β -arrestin2-deficient mice. Data are mean \pm SEM in control or nicotinic acid-treated animals ($n = 4$ –10 per condition).

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_i/G_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 induce cutaneous flushing (19, 27, 28). We wondered whether biased signaling toward G_i/G_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 Luc- β -arr-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 non-flushing 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 con-

sistent 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.

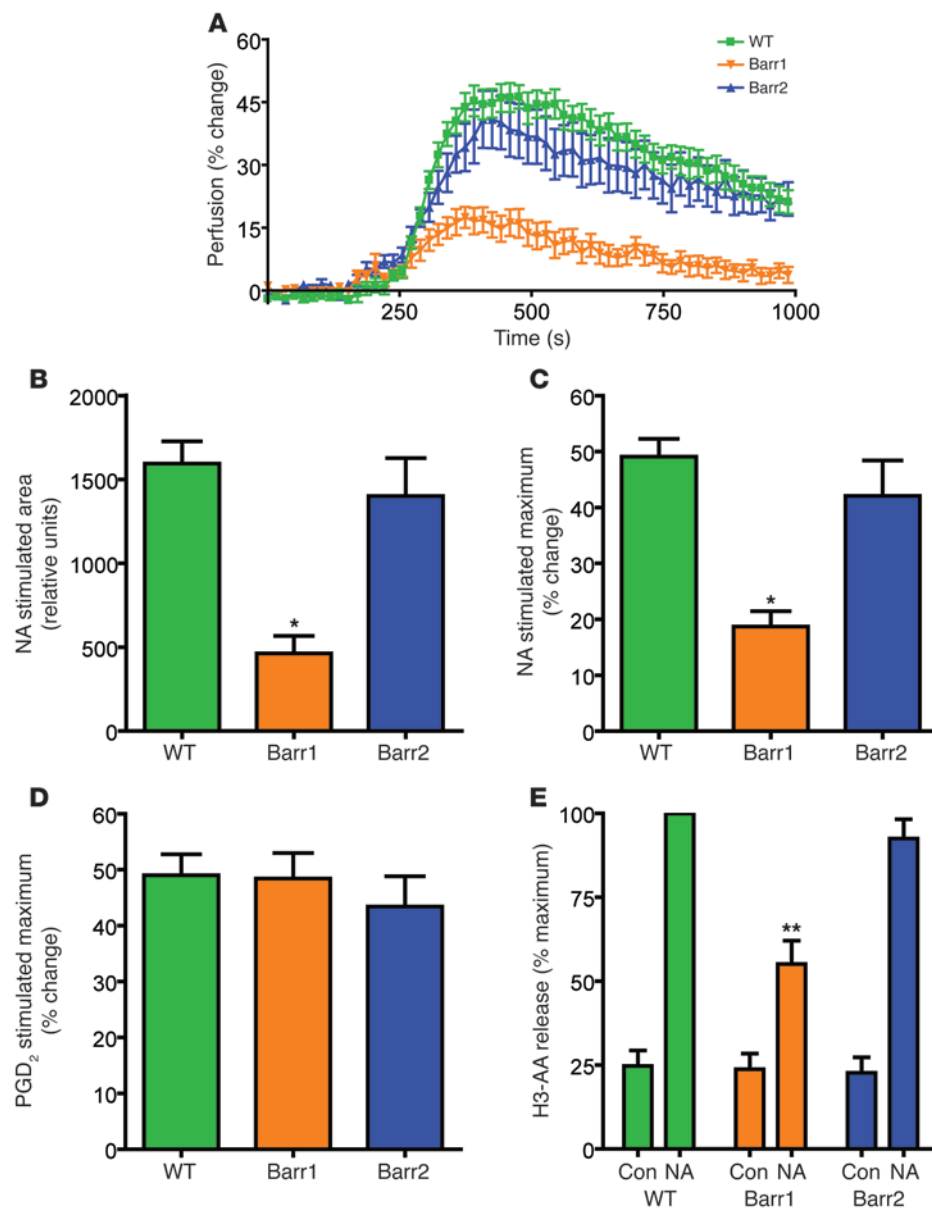
Discussion

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_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 8**

Nicotinic acid–induced cutaneous flushing and activity of cPLA₂ is attenuated in β -arrestin1–deficient mice. **(A)** Perfusion of the ventral ear in wild-type, β -arrestin1–deficient, or β -arrestin2–deficient mice was measured with laser Doppler. Baseline perfusion was measured for 150 seconds, then mice were given i.p. injections of 100 mg/kg nicotinic acid. Data are mean \pm SEM for change in perfusion as a function of time. **(B)** Total response to nicotinic acid, plotted as mean \pm SEM area under the curve. **(C)** Maximum response to nicotinic acid, plotted as mean \pm SEM. $*P < 0.0001$ versus wild type. **(D)** Maximum response to PGD₂, plotted as mean \pm SEM ($n = 15$ – 25 per condition). **(E)** Nicotinic acid–stimulated release of eicosanoids was measured in peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were loaded with H³-arachidonic acid (H³-AA) for 24 hours, and then rinsed to remove arachidonic acid not incorporated into cell membrane lipids. Macrophages were stimulated for 10 minutes with 200 μ M nicotinic acid, and radioactivity released into the media was measured. Data are mean \pm SEM ($n = 4$ per condition) for change in radioactivity, plotted as a percentage of the maximum response. $**P = 0.0001$ versus nicotinic acid–treated wild type.

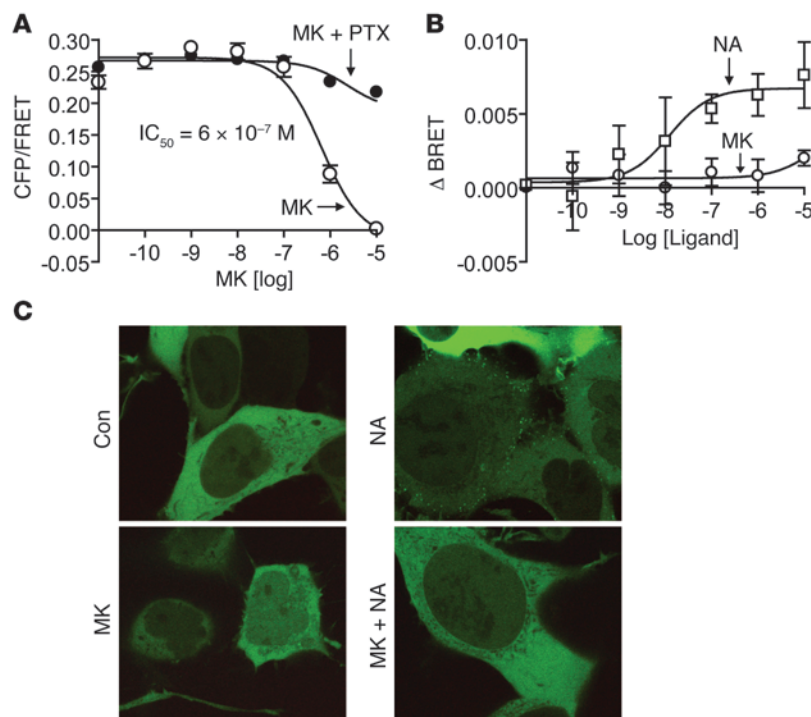


the beneficial effects of GPR109A signaling while avoiding signaling pathways that mediate cutaneous flushing.

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.

**Figure 9**

MK-0345-induced G protein signaling, β -arrestin conformational changes, and recruitment. **(A)** Cells expressing GPR109A and the ICUE2 biosensor were treated with forskolin and MK-0345 (MK). MK-0345 (open circles) decreased cAMP in a dose-dependent fashion, and this response was inhibited by pertussis toxin (filled circles). **(B)** Cells expressing GPR109A and the BRET reporter Luc- β -arr-YFP were treated with nicotinic acid (open squares) or MK-0345 (open circles). MK-0345 failed to induce conformational changes in β -arrestin2. Data are mean \pm SEM of 3 independent experiments. **(C)** Cells expressing GPR109A β -arrestin1-mYFP were treated with nicotinic acid, MK-0345, or both. Prior to nicotinic acid stimulation, β -arrestin1 resided in the cytosol; it translocated to bind GPR109A in the membrane in response to 10 μ M nicotinic acid. No translocation was noted in cells stimulated with 200 μ M MK-0345 or in cells treated with 10 μ M nicotinic acid in the presence of 200 μ M MK-0345. Images are representative of 4 independent experiments. Original magnification, $\times 100$.

Methods

Materials. Nicotinic acid, PGD₂, and (2-hydroxypropyl)- β -cyclodextrin were obtained from Sigma-Aldrich. MK-0345 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 -arr-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% 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 μ g plasmid in a 10-cm tissue culture plate. Cells expressing GPR109A alone were selected with 400 μ g/ml G418 (Sigma-Aldrich), and colonies of stable transfectants were isolated. Cells expressing GPR109A in combination with the ICUE2 biosensor were selected with 400 μ g/ml G418 and 300 μ g/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-0345 for 3 minutes, followed by stimulation with 10 μ M for-

skolin 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 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-0345, 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 μ M 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 remove unbroken cells and nuclear 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 wall, 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 μ M) 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 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 μ M 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 -arr was expressed alone.

Immunoblotting and immunoprecipitation. Phosphorylated ERK immunoblotting using the antibody anti-phospho-p44/42 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 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 of β -arrestin1 and β -arrestin2 was confirmed by immunoblotting. Only experiments with confirmed protein silencing were analyzed.

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 ear 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 \times 5-mm image size, a 10-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 PGD₂ dissolved in PBS. For eicosanoid release assays, thioglycollate-elicited peritoneal macrophages were collected as previously described (46, 47). Cells were pretreated with 0.1 mCi/ml H³-arachidonic acid for 24 hours, then rinsed 5 times to remove unincorporated H³-arachidonic acid (48). Macrophages (1 \times 10⁶ cells/well) were stimulated for 10 minutes with 200 μ M 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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- DeWire, S.M., Ahn, S., Lefkowitz, R.J., and Shenoy, S.K. 2007. Beta-arrestins and cell signaling. *Annu. Rev. Physiol.* **69**:483–510.
- Krupnick, J.G., and Benovic, J.L. 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* **38**:289–319.
- Lefkowitz, R.J., Rajagopal, K., and Whalen, E.J. 2006. New roles for beta-arrestins in cell signaling: not just for seven-transmembrane receptors. *Mol. Cell.* **24**:643–652.
- Pike, N.B. 2005. Flushing out the role of GPR109A (HM74A) in the clinical efficacy of nicotinic acid. *J. Clin. Invest.* **115**:3400–3403.
- Canner, P.L., et al. 1986. Fifteen year mortality in Coronary Drug Project patients: long-term benefit with niacin. *J. Am. Coll. Cardiol.* **8**:1245–1255.
- Benyo, Z., et al. 2005. GPR109A (PUMA-G/HM74A) mediates nicotinic acid-induced flushing. *J. Clin. Invest.* **115**:3634–3640.
- Benyo, Z., Gille, A., Bennett, C.L., Clausen, B.E., and Offermanns, S. 2006. Nicotinic acid-induced flushing is mediated by activation of epidermal langerhans cells. *Mol. Pharmacol.* **70**:1844–1849.
- Morrow, J.D., Awad, J.A., Oates, J.A., and Roberts, L.J., 2nd. 1992. Identification of skin as a major site of prostaglandin D2 release following oral administration of niacin in humans. *J. Invest. Dermatol.* **98**:812–815.
- Andersson, R.G., Aberg, G., Brattsand, R., Ericsson, E., and Lundholm, L. 1977. Studies on the mechanism of flush induced by nicotinic acid. *Acta Pharmacol. Toxicol. (Copenh.)* **41**:1–10.
- Eklund, B., Kaijser, L., Nowak, J., and Wennmalm, A. 1979. Prostaglandins contribute to the vasodilation induced by nicotinic acid. *Prostaglandins*. **17**:821–830.
- Lai, E., et al. 2007. Suppression of niacin-induced vasodilation with an antagonist to prostaglandin D2 receptor subtype 1. *Clin. Pharmacol. Ther.* **81**:849–857.
- Lin, F.T., et al. 1997. Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1. *J. Biol. Chem.* **272**:31051–31057.
- Lefkowitz, R.J., and Shenoy, S.K. 2005. Transduction of receptor signals by beta-arrestins. *Science*. **308**:512–517.
- Tunaru, S., et al. 2003. PUMA-G and HM74 are receptors for nicotinic acid and mediate its antilipolytic effect. *Nat. Med.* **9**:352–355.
- Bohn, L.M., Gainetdinov, R.R., Lin, F.T., Lefkowitz, R.J., and Caron, M.G. 2000. Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature*. **408**:720–723.
- Bohn, L.M., Lefkowitz, R.J., and Caron, M.G. 2002. Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. *J. Neurosci.* **22**:10494–10500.
- Bohn, L.M., et al. 1999. Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science*. **286**:2495–2498.



18. Raehal, K.M., Walker, J.K., and Bohn, L.M. 2005. Morphine side effects in beta-arrestin 2 knockout mice. *J. Pharmacol. Exp. Ther.* **314**:1195–1201.
19. Richman, J.G., et al. 2007. Nicotinic acid receptor agonists differentially activate downstream effectors. *J. Biol. Chem.* **282**:18028–18036.
20. Sakai, T., Kamanna, V.S., and Kashyap, M.L. 2001. Niacin, but not gemfibrozil, selectively increases LP-AI, a cardioprotective subfraction of HDL, in patients with low HDL cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **21**:1783–1789.
21. Charest, P.G., Terrillon, S., and Bouvier, M. 2005. Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. *EMBO Rep.* **6**:334–340.
22. Cheng, Z.J., et al. 2000. beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J. Biol. Chem.* **275**:2479–2485.
23. Kohout, T.A., et al. 2004. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J. Biol. Chem.* **279**:23214–23222.
24. Lin, L.L., et al. 1993. cPLA2 is phosphorylated and activated by MAP kinase. *Cell.* **72**:269–278.
25. Xiao, K., et al. 2007. Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc. Natl. Acad. Sci. U. S. A.* **104**:12011–12016.
26. Kather, H., Aktories, K., Schulz, G., and Jakobs, K.H. 1983. Islet-activating protein discriminates the antilipolytic mechanism of insulin from that of other antilipolytic compounds. *FEBS Lett.* **161**:149–152.
27. Lai, E., et al. 2008. Effects of niacin receptor partial agonist, MK-0354, on plasma free fatty acids, lipids, and cutaneous flushing in humans. *J. Clin. Lipidol.* **2**:375–383.
28. Semple, G., et al. 2008. 3-(1H-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (MK-0354): a partial agonist of the nicotinic acid receptor, G-protein coupled receptor 109a, with antilipolytic but no vasodilatory activity in mice. *J. Med. Chem.* **51**:5101–5108.
29. Guyton, J.R. 2007. Niacin in cardiovascular prevention: mechanisms, efficacy, and safety. *Curr. Opin. Lipidol.* **18**:415–420.
30. Wise, A., et al. 2003. Molecular identification of high and low affinity receptors for nicotinic acid. *J. Biol. Chem.* **278**:9869–9874.
31. Ganji, S.H., Kamanna, V.S., and Kashyap, M.L. 2003. Niacin and cholesterol: role in cardiovascular disease. *J. Nutr. Biochem.* **14**:298–305.
32. Jin, F.Y., Kamanna, V.S., and Kashyap, M.L. 1999. Niacin accelerates intracellular ApoB degradation by inhibiting triacylglycerol synthesis in human hepatoblastoma (HepG2) cells. *Arterioscler. Thromb. Vasc. Biol.* **19**:1051–1059.
33. Jin, F.Y., Kamanna, V.S., and Kashyap, M.L. 1997. Niacin decreases removal of high-density lipoprotein apolipoprotein A-I but not cholesterol ester by Hep G2 cells. Implication for reverse cholesterol transport. *Arterioscler. Thromb. Vasc. Biol.* **17**:2020–2028.
34. Drake, M.T., et al. 2008. beta-arrestin-biased agonism at the beta2-adrenergic receptor. *J. Biol. Chem.* **283**:5669–5676.
35. Gesty-Palmer, D., et al. 2006. Distinct beta-arrestin and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J. Biol. Chem.* **281**:10856–10864.
36. Wei, H., et al. 2003. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc. Natl. Acad. Sci. U. S. A.* **100**:10782–10787.
37. Wisler, J.W., et al. 2007. A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **104**:16657–16662.
38. Kenakin, T. 2007. Functional selectivity through protean and biased agonism: who steers the ship? *Mol. Pharmacol.* **72**:1393–1401.
39. Violin, J.D., Ren, X.R., and Lefkowitz, R.J. 2006. G-protein-coupled receptor kinase specificity for beta-arrestin recruitment to the beta2-adrenergic receptor revealed by fluorescence resonance energy transfer. *J. Biol. Chem.* **281**:20577–20588.
40. Meyers, C.D., Liu, P., Kamanna, V.S., and Kashyap, M.L. 2007. Nicotinic acid induces secretion of prostaglandin D2 in human macrophages: an in vitro model of the niacin flush. *Atherosclerosis.* **192**:253–258.
41. DiPilato, L.M., Cheng, X., and Zhang, J. 2004. Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc. Natl. Acad. Sci. U. S. A.* **101**:16513–16518.
42. Violin, J.D., et al. 2008. beta2-adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics. *J. Biol. Chem.* **283**:2949–2961.
43. Attramadal, H., et al. 1992. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *J. Biol. Chem.* **267**:17882–17890.
44. Ahn, S., Nelson, C.D., Garrison, T.R., Miller, W.E., and Lefkowitz, R.J. 2003. Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. *Proc. Natl. Acad. Sci. U. S. A.* **100**:1740–1744.
45. Haqq, A.M., et al. 2005. The Study of the Effects of Diet on Metabolism and Nutrition (STEDMAN) weight loss project: Rationale and design. *Contemp. Clin. Trials.* **26**:616–625.
46. Misra, U.K., and Pizzo, S.V. 2000. Cytosolic phospholipase A(2) activity associated with nuclei is not inhibited by arachidonyl trifluoromethyl ketone in macrophages stimulated with receptor-recognized forms of alpha(2)-macroglobulin. *Arch. Biochem. Biophys.* **379**:153–160.
47. Misra, U.K., and Pizzo, S.V. 2002. Regulation of cytosolic phospholipase A2 activity in macrophages stimulated with receptor-recognized forms of alpha 2-macroglobulin: role in mitogenesis and cell proliferation. *J. Biol. Chem.* **277**:4069–4078.
48. Levine, L. 2003. Tamoxifen stimulates arachidonic acid release from rat liver cells by an estrogen receptor-independent, non-genomic mechanism. *BMC Cancer.* **3**:24.