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β -Arrestins specifically constrain β_2 -adrenergic receptor signaling and function in airway smooth muscle

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Abstract

Chronic use of inhaled beta-agonists by asthmatics is associated with a loss of bronchoprotective effect and deterioration of asthma control. Beta-agonist-promoted desensitization of airway smooth muscle beta-2-adrenergic receptors, mediated by G protein-coupled receptor kinases and arrestins, is presumed to underlie these effects, but such a mechanism has never been demonstrated. Using *in vitro*, *ex vivo*, and *in vivo* murine models, we demonstrate that beta-arrestin-2 gene ablation augments beta-agonist-mediated airway smooth muscle relaxation, while augmenting beta-agonist-stimulated cyclic adenosine monophosphate production. In cultures of human airway smooth muscle, small interfering RNA-mediated knockdown of arrestins also augments beta-agonist-stimulated cyclic adenosine monophosphate production. Interestingly, signaling and function mediated by m2/m3 muscarinic acetylcholine receptors or prostaglandin E₂ receptors were not affected by either beta-arrestin-2 knockout or arrestin knockdown. Thus, arrestins are selective regulators of beta-2-adrenergic receptor signaling and function in airway smooth muscle. These results and our previous findings, which demonstrate a role for arrestins in the development of allergic inflammation in the lung, identify arrestins as potentially important therapeutic targets for obstructive airway diseases.—Deshpande, D. A., Theriot, B. S., Penn, R. B., Walker, J. K. L. β -Arrestins specifically constrain β_2 -adrenergic receptor signaling and function in airway smooth muscle.

Beta-agonists, *via* activation of the beta-2-adrenergic receptor (β_2 AR), antagonize airway smooth muscle (ASM) contraction and are important therapeutic agents in the management of obstructive airway diseases such as asthma (1). However, many asthmatics are not sufficiently managed by beta-agonist therapy, and continuous use of inhaled beta-agonist has been shown to result in a loss of bronchoprotective effect (2–4) and deterioration of asthma control (5).

One of the potential mechanisms that limit the therapeutic efficacy of beta-agonists is desensitization of ASM β_2 ARs. β_2 AR desensitization has been shown to occur in numerous cell types following either acute or chronic exposure to beta-agonists. Studies examining recombinant β_2 ARs expressed in cell lines, as well as a handful of studies examining endogenous β_2 ARs in primary cell types, have demonstrated important roles for G protein-coupled receptor (GPCR) kinases (GRKs) and arrestins in mediating β_2 AR desensitization to beta-agonists (as reviewed in refs. 1, 6, 7). GRKs phosphorylate the β_2 AR when it is

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occupied by beta-agonists, and arrestins subsequently bind the phosphorylated receptor, effectively blocking receptor interaction with, and activation of, the heterotrimeric G protein G_s . This quenching or “arresting” of β_2AR signaling occurs rapidly and also initiates a process of receptor internalization. With chronic exposure to beta-agonists, arrestins help to direct internalized β_2AR s to lysosomes, where they are degraded, such that new β_2AR synthesis is required to ultimately recover from chronic desensitization (8).

The extent to which GRK- and arrestin-dependent β_2AR desensitization occurs in ASM and affects the functional response to beta-agonists is unclear. We have previously determined that overexpression of β -arrestin (β arr) -1 or β arr-2 (also termed arrestin2 and arrestin3, respectively) in human ASM cultures causes β_2AR desensitization and attenuation of beta-agonist-stimulated signaling (9). In a murine model of ovalbumin-induced allergic inflammation, we have also observed that ablation of the β arr-2 gene causes a significant inhibition of inflammatory cell influx into allergen-challenged murine airway and prevents airway hyperresponsiveness (10). Whether manipulation of arrestin expression within physiological ranges affects β_2AR ASM signaling or function in either cell-based or *in vivo* assays has yet to be determined.

In the current study, we examined the selectivity and efficacy of β arr-1/2 knockdown and specific β arr-2 knockout in regulating GPCR function in *in vitro*, *ex vivo*, and *in vivo* analyses of ASM. Results demonstrate that arrestins are selective and efficacious modulators of beta-agonist-mediated signaling and function in ASM, thus identifying them as a potentially important therapeutic target for obstructive airway diseases.

MATERIALS AND METHODS

Generation of β arr-2-knockout (β arr-2-KO) mice

β arr-2-KO mice were generated as described previously (11) and backcrossed for six generations onto the C57BL/6 background. β arr-2-KO and wild-type (WT) sibling pairs aged 10–16 wk were used for *in vivo* measurement of lung function, *ex vivo* measurement of tracheal contraction, and isolation of ASM cells under protocols approved by the Animal Care and Use Committees at Duke University and Wake Forest University Health Sciences. β arr-2 gene ablation was confirmed by triplex polymerase chain reaction (PCR).

Immunoblotting

Mouse lung was pulverized in liquid nitrogen and solubilized in radioimmunoprecipitation assay (RIPA) buffer. Tissue lysates were buffered with Laemmli solution and sonicated, and equal amounts of total protein (as measured by a DC protein assay read at 750 nm; Bio-Rad, Hercules, CA, USA) were loaded and separated on 10% Tris-glycine polyacryl-amide gels (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to nitrocellulose membranes for immunoblotting. β -Arrestins were detected with the polyclonal Ab A2CT (generous gift from Robert J. Lefkowitz, Duke University, Durham, NC, USA). A2CT preferentially recognizes β arr-2 over β arr-1. Chemiluminescent detection was performed with horseradish peroxidase-coupled secondary Ab (Amersham Biosciences, Pittsburgh, PA, USA) and SuperSignal West Femto reagent (Pierce, Rockford, IL, USA). Chemiluminescence was quantified by a charge-coupled device camera (Syngene, Frederick, MD, USA); representative images are shown as inverted grayscale. For immunoblots of lysates from cultured human ASM cells, blots were probed with Arr178 antibody (generous gift from Jeffrey L. Benovic, Thomas Jefferson University, Philadelphia, PA, USA) and an IRDye 800 secondary antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) conjugated with an infrared fluorophore. Bands were visualized and signals (infrared emission)

quantified directly using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described previously (12).

Mouse lung and ASM membrane preparation

To prepare ASM membranes, tracheae and bronchi were dissected free of loose connective tissue and lung parenchyma, and the epithelium was denuded. The tissue was placed in ice-cold buffer (25 mM Tris-HCl, pH 7.4, and 5 mM EDTA, pH 8.0) containing protease inhibitors and homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY) at setting 8 for 3×10 s periods. The resulting homogenate was spun at 250 *g* for 10 min at 4°C, the low-speed pellet was discarded, and the supernatant was centrifuged at 18,000 rpm for 20 min at 4°C. The pellet was resuspended in binding buffer (75 mM Tris-HCl, 2 mM EDTA, 12.5 mM MgCl₂) at a concentration of 0.5 to 1.0 mg protein/ml (protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard). Membranes from peripheral lung were similarly prepared after dissecting away major airways and blood vessels.

β-AR radioligand binding

β-AR radioligand binding studies were performed in fresh lung and ASM membranes by use of the nonselective β-AR antagonist [¹²⁵I]-labeled cyanopindolol (¹²⁵I-CYP), as described previously (13). Maximal binding (B_{\max}) was measured by use of a saturating amount of ¹²⁵I-CYP on 20 μg of membrane protein, incubating 40 min at 37°C. Inclusion of 25 μM propranolol defined nonspecific binding. Assays were stopped by dilution with 5 ml of ice-cold binding buffer and rapid filtration over glass fiber filters, followed by four 5-ml washes, before counting in a gamma counter. Total binding represented <10% of ¹²⁵I-CYP counts added. Specific binding, which was calculated as the difference between triplicate averages of total and nonspecific counts bound to the filters, comprised on average 64% and 96% of total binding for ASM and lung, respectively.

Lung function measurements

Contractile and relaxation responsiveness of airways to methacholine and albuterol were determined as described previously (14). Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) diluted 50% with saline, then surgically prepared with a tracheal cannula and a jugular vein catheter. Mice were paralyzed with pancuronium bromide (0.25 mg/kg) and ventilated with 100% oxygen at a constant volume of 6–8 ml/kg and a frequency of 165 breaths/min. These ventilator settings resulted in an average resting peak tracheal pressure of 6.5 ± 0.1 cm H₂O and have been previously shown to provide normal arterial blood gases. Airway pressure was measured at a side port of the tracheal cannula connected to a Validyne differential pressure transducer (Validyne Engineering, Northridge, CA, USA). The time-integrated change in peak tracheal pressure, or airway pressure time index (APTI) (15, 16), was calculated for a 60 s period from the point at which tracheal pressure began to increase in response to methacholine (MCh).

Anesthetized and paralyzed mice were intravenously administered 50 μg/kg MCh as a contractile priming dose. This was followed by two consecutive intravenous administrations of 100 μg/kg MCh alone and administration of a combined preparation of either 100 μg/kg MCh and 30 μg/kg albuterol or 100 μg/kg MCh and 5 μg/kg prostaglandin E₂ (PGE₂). Each drug administration was separated by a 5-min interval. The response to MCh in the presence of albuterol or PGE₂ was reduced compared to the contractile response to MCh alone, and the percent change was calculated and used to determine the net decrease in the MCh-induced airway response.

Ex vivo ASM tension development

Murine tracheae excised after euthanasia by CO₂ inhalation were cleaned of surrounding connective tissue and mounted into a multiwire myograph chamber in Krebs-Henseleit solution (pH 7.40–7.45) maintained at 37°C with 5% CO₂ and 95% O₂, with frequent changing of the solution. The chambers were mounted onto the Myo-Interface (model 610 M) and connected to the transducer and PowerLab (ADInstruments, Colorado Springs, CO, USA) for data transferring and recording. Chart5 software for Windows (ADInstruments) was used to collect and analyze the data. A basal tension of 0.5 g was set, and the tracheal rings were stimulated with 10 μM MCh for 5 min. The rings were washed, and preload was reset to 0.5 g for 30 min. After stabilization, the tracheal rings were stimulated with 10 μM MCh and allowed to contract for 10 min. Stimulation of the rings with 10 μM MCh produces ~80% maximum tension in the rings, as determined previously (15). Subsequently, the precontracted rings were stimulated with increasing concentrations of isoproterenol (ISO) or PGE₂ for 5 min and tension was recorded. In a select set of experiments, the precontracted rings were stimulated with 10 μM forskolin (FSK). In another set of experiments, the rings were contracted with 60 mM KCl for 5 min and subsequently stimulated with 1 μM MCh for 5 min. The net contractile responses were determined by subtracting the basal tension from that of the peak tension on agonist stimulation, and the change in the tension was normalized to the wet weight of the tracheal ring segment. Relaxation responses were calculated as percentage change in MCh-induced tension on stimulation with different concentrations of relaxant agents.

Generation of murine and human ASM cultures

Human ASM cell cultures were generated from tissue obtained at autopsy, as described previously (13), and cultures in passages 5–7 were examined. For generation of murine ASM cultures, tracheae harvested from 5 mice were cleaned of surrounding tissues and pooled to isolate ASM cells using the above procedure for human ASM cultures with minor modifications, as described recently (17). ASM cells were characterized by immunostaining using anti- α -actin and anti-smooth muscle myosin heavy-chain antibodies.

Small interfering (si)RNA-mediated knockdown of β -arrestins in ASM

siRNA oligos were designed based on human β arr-1/2 nucleotide sequence (sense, 5' ACC UGC GCC UUC CGC UAU GTT 3' antisense, 5' CAU AGC GGA AGG CGC AGG UTT 3'), as described previously (18). Control (scrambled) or β arr-1/2 siRNA oligos were annealed at 37°C for 1 h, and 5 μg of the annealed oligo mixtures was used to transfect HASM cells by electroporation (Amaza Biosystems, Gaithersburg, MD, USA), as described previously (13). Twenty-four hours after transfection, the cells were replated for subsequent assessment of β arr-1/2 protein expression by immunoblot analysis.

Measurement of intracellular cAMP accumulation, phosphoinositide hydrolysis

For analyses of regulation of intracellular cAMP accumulation, murine or human ASM cells plated in 24-well plates were grown to near confluence and serum starved for 24 h. The cells were stimulated with various concentrations of ISO, PGE₂, or FSK in phosphate-buffered saline containing 300 μM ascorbic acid and 1 mM RO-20-1724 (phosphodiesterase inhibitor) at 37°C for 0–10 min. cAMP was extracted and quantified by RIA using ¹²⁵I-cAMP and anti-cAMP antibody, as described previously (13). All conditions were assayed in triplicate wells and duplicate RIA tubes. Analysis of agonist-stimulated phosphoinositide (PI) hydrolysis in murine ASM cultures was performed as described previously (17). Cells were plated and serum starved as above and loaded with 2 μCi/ml myo-[³H]inositol for 18 h. After washing with phosphate-buffered saline, the cells were incubated with DMEM containing 5 mM LiCl for 10 min, then stimulated with vehicle or the muscarinic receptor

agonist carbachol (CCh; 10 μ M) for 30 min. Reactions were quenched with 20 mM formic acid, and inositol and phosphoinositide (PI) fractions were separated by anion exchange chromatography. PI production was calculated by dividing PI fraction by total (PI + inositol) inositol fraction, and the data were reported as fold basal (agonist-stimulated/vehicle). PI hydrolysis was not examined in human ASM cells due to the rapid loss of m3 mAChRs that occurs with culture (19).

Statistical analysis

Data analysis was performed using GraphPad Prism (San Diego, CA, USA), and data are expressed as means \pm SE. Group comparisons were performed using 2-way ANOVA or Student's *t* test where appropriate, with a value of *P* < 0.05 sufficient to reject the null hypothesis.

RESULTS

Arrestin knockout and knockdown in ASM cells enhances β -AR signaling

To assess arrestin-dependent regulation of β -AR signaling in ASM cells, ASM cultures derived from β arr-2-KO mice tracheae and human ASM cultures derived from multiple donors were generated. As shown in Fig. 1A, β arr-1 and β arr-2 are both expressed in WT mouse lung, whereas β arr-2-KO mice lack the faster migrating (~46.3 kDa predicted and 55 kDa observed, β arr-2) band in immunoblots. Relative to that observed for ASM cultures derived from WT mice, cAMP accumulation in β arr-2-KO ASM cultures in response to ISO stimulation was significantly greater at all concentrations 10 nM tested (Fig. 1B, C). FSK-stimulated cAMP accumulation was unaffected by β arr-2 knockout, suggesting differences in ISO-stimulated cAMP accumulation were a result of regulation of the β_2 AR locus. Conversely, PGE₂ stimulated a low level of cAMP accumulation that was similar in both WT and β arr-2-KO ASM cultures (data not shown), and β arr-2 knockout also failed to affect phosphoinositide hydrolysis stimulated by mAChR activation (Fig. 1D).

To determine whether β_2 AR signaling was similarly regulated by arrestins in human ASM, five distinct cultures derived from five different donor tracheae were transfected with siRNA oligonucleotides targeting β arr-1 and -2. Immunoblot analysis of culture lysates harvested 96 h after transfection reveals significant knockdown of arrestin expression relative to that in control cells transfected with control oligos (Fig. 2A). β arr-1 expression is readily detected in human ASM cultures and is significantly reduced (73 \pm 2%, *n*=5) by siRNA-mediated knockdown. β arr-2 expression is either absent or at the limit of detection in human ASM cultures; a faint, faster migrating band (relative to the β arr-1 band) is suggested in some blots but is not consistently recognized whether blots are probed with Arr178 Ab or more β arr-2-selective antibodies (not shown). cAMP accumulation stimulated by submaximal concentrations of beta-agonist was not significantly affected by arrestin knockdown (not shown) but that stimulated by 1 μ M ISO was significantly increased (Fig. 2B). Conversely, PGE₂-stimulated cAMP accumulation in human ASM cultures was slightly inhibited by arrestin knockdown, and FSK-stimulated cAMP accumulation was unaffected (Fig. 2B).

Arrestin knockout augments relaxant effect of beta-agonist on contracted ASM *ex vivo* and *in vivo*

To assess arrestin-dependent regulation of β_2 AR-mediated function in ASM, regulation of ASM contraction in excised tracheal rings was characterized. Tension generation stimulated by MCh was similar in rings from WT and β arr-2-KO mice, as was KCl-mediated contraction (Fig. 3A). However, ISO-mediated relaxation of MCh-induced contraction was significantly greater in rings from β arr-2-KO mice compared to that in WT rings (Fig. 3B).

Conversely, PGE₂- and FSK-mediated relaxation was not significantly different between groups.

Regulation of β_2 AR function *in vivo* was characterized by assessing albuterol-mediated changes in MCh-stimulated airway responsiveness. MCh challenge increased airway responsiveness (APTI) to a similar degree in both WT and β arr-2-KO mice (52.1 ± 5.5 vs. 48.4 ± 3.0 cm H₂O \times s in WT and β arr-2-KO groups, respectively). Albuterol (30 μ g/kg) treatment significantly inhibited MCh-induced increases in airway responsiveness for both WT and β arr-2-KO mice. However, this reduction was significantly greater in β arr-2-KO mice compared to that in WT controls (Fig. 4). Conversely, PGE₂ treatment significantly reduced MCh-induced airway responsiveness, but β arr-2 knockout did not alter this effect.

Arrestin knockout does not alter lung or ASM β -AR density

We examined total β -AR density (Table 1). The maximum concentration of β -AR binding sites (B_{max}) to tracheal smooth muscle membranes was not different between WT and β arr-2-KO mice (80.3 ± 25.2 and 88.9 ± 13.8 fmol/mg protein, respectively). Similarly, WT and β arr-2-KO mice expressed similar levels of β -AR in whole lung membranes (444 ± 56 and 471 ± 38 fmol/mg protein, respectively).

DISCUSSION

This study is the first to demonstrate the efficacy and selectivity of arrestins in regulating GPCR function in ASM. In both *in vivo* and *ex vivo* murine models of ASM contractile regulation, β arr-2 is shown to constrain beta-agonist-mediated (β_2 AR-dependent) antagonism of ASM contraction, while not affecting antagonism mediated by PGE₂ (EP2/4R-dependent) or the contractile effect of methacholine (m3 mAChR-dependent). These *in vivo* and *ex vivo* data are consistent with *in vitro* cell data, which demonstrate increased β -agonist-mediated cAMP accumulation in cells lacking β arr-2. Taken together, these data demonstrate that the classic desensitization of β_2 AR signaling involving β -arrestins is functionally relevant with respect to bronchorelaxation in the whole animal.

The “arresting” function of β -arrestin was first demonstrated for the β_2 AR by Benovic *et al.* (20). The canonical β_2 AR signaling pathway involves agonist-induced G_{α_s} activation, stimulation of adenylyl cyclase (AC), cAMP accumulation, and PKA activation. In the case of ASM, PKA phosphorylates several target proteins to promote ASM relaxation (reviewed in ref. 1). This pathway is desensitized by β -arrestin binding to the agonist-activated, GRK-phosphorylated receptor, which sterically interdicts further receptor- G_{α_s} coupling (21). In the present study, we measured cAMP accumulation as an indicator of receptor- G_{α_s} -AC signaling. Using primary murine ASM cultures, we demonstrate that isoproterenol-, but not PGE₂- or forskolin-induced cAMP accumulation is increased in cells lacking β arr-2 relative to WT cells. Similar results were obtained in human ASM cells (which express predominantly β arr-1) when β arr-1 and β arr-2 levels were knocked down by transfection of siRNA. Because forskolin raises cAMP levels in ASM through receptor-independent activation of AC (22), the lack of effect of arrestin knockout or knockdown of forskolin-stimulated cAMP accumulation suggests β -arrestins regulate signaling at the receptor locus. The lack of effect on PGE₂-mediated signaling and function further suggests that neither G_{α_s} nor the EP2 receptor (EP2R) is regulated by β -arrestins in ASM.

These results, demonstrating the lack of effect of arrestin knockout/knockdown on PGE₂ effects on ASM, are in agreement with our previous *in vitro* data that has demonstrated that the EP2R is the functionally dominant EP receptor expressed in ASM and that β -arrestins do not translocate to activated EP2R, arrest EP2R- G_{α_s} signaling, or promote EP2R internalization (9). Thus, the EP2R is an example of one GPCR whose signaling and

internalization are not regulated by β -arrestins, presumably a consequence of the EP2R being a poor substrate for GRKs.

Our finding that MCh-induced airway responsiveness and ASM contraction *ex vivo* were not affected by β arr-2 knockout identifies the m3 mAChR (the principal effector of ASM contraction mediated by mAChR agonists in the mouse; ref. 23) as an additional GPCR whose function is not regulated by arrestins. Consistent with these data, we also failed to observe differences in MCh-mediated phosphoinositide hydrolysis between cultures of ASM from β arr-2-KO and WT mice. Although these are the first data assessing arrestin-dependent regulation of endogenously expressed m3 mAChR, previous studies have demonstrated that although GRKs can phosphorylate purified, reconstituted m3 mAChR (24), agonist-induced internalization of overexpressed m3 mAChRs in human embryonic kidney cells is not affected by overexpressed or dominant-negative arrestins (25).

The aforementioned actions of β -arrestins occur within seconds to minutes of agonist-receptor binding and demonstrate a physiological mechanism by which β_2 AR-mediated relaxation of ASM is innately limited. Steric inhibition of β_2 AR-G α s coupling by β -arrestins, relieved by β -arrestin knockout or knockdown, is known to mediate rapid β_2 AR desensitization. In numerous cell-based studies, a more delayed β -arrestin-mediated regulation of the β_2 AR is known to occur as a result of the dependence of receptor internalization on β -arrestin function (6). β -arrestin acts as an adaptor protein that links the β_2 AR to clathrin-mediated internalization. Once internalized, β_2 ARs are directed to acidic endosomes, where they are dephosphorylated and recycled as competent receptors to the cell membrane or directed to lysosomes where they are degraded and thus, down-regulated (8, 26). β -arrestin regulation of internalization and/or receptor recycling did not likely contribute to the beta-agonist effects observed in this study, since experiments assessing cAMP accumulation and airway contractile responsiveness were of short duration. Moreover, the similarity in lung and ASM β -AR density between WT and β arr-2-KO mice suggests that chronic up-regulation of β -ARs does not account for the effect of arrestins on beta-agonist regulation of airway responsiveness. Future studies examining the effects of chronic beta-agonist treatment should help elucidate the role of arrestin-dependent β_2 AR trafficking in modulating the β_2 AR function in ASM.

The ability of arrestin knockout or knockdown to augment signaling and functional consequences of β_2 AR activation occurs against a backdrop of other regulatory mechanisms also capable of desensitizing β_2 AR signaling. GRKs have been shown sufficient to mediate agonist-induced β_2 AR desensitization (21, 27), and PKA-mediated phosphorylation of the β_2 AR (28) has also been shown to be an important feedback regulator of the β_2 AR in many cell types. Because these mechanisms are known to be active in ASM (ref. 13 and unpublished results), our findings suggest that they cannot fully compensate for a loss of arrestins and suggest a clear physiological role for arrestins in ASM.

The observation that knockout of β arr-2 in the mouse is sufficient to augment β_2 AR-mediated signaling and function in ASM is consistent with the findings of Oakley *et al.* (29), which demonstrate that the β_2 AR binds to β arr-2 more readily and to a greater extent than it does β arr-1. That β arr-1 is unable to fully compensate for a loss of β arr-2 suggests that these differences in binding kinetics and affinity are of physiological relevance. However, in human ASM cell cultures, which express β arr-1 but have at most extremely low levels of β arr-2, knockdown of β arr-1/2 was able to significantly augment β_2 AR signaling. This finding suggests that β arr-1-dependent regulation of the β_2 AR is also of physiological consequence in ASM. Because knockout of both β arr-1 and β arr-2 is lethal (reviewed in ref. 30), we are unable to judge the relative importance of each arrestin on our experimental outcomes.

Nevertheless, our findings point to β -arrestins as important regulators of ASM function and implicate agonist-specific (homologous) desensitization of ASM β_2 ARs as important in airway physiology, and possibly, airway disease. Although the significance of endogenous catecholamines on ASM contraction is unclear, most asthmatics control their symptoms through the use of inhaled beta-agonists. Both short- and long-acting β -agonists are prescribed with caution as it is generally accepted that their chronic use is associated with a loss of bronchoprotection and a loss of asthma control associated with increased risk of exacerbations and death (31–35). The mechanisms underlying worsening of asthma severity with chronic use of beta-agonists have not been clearly established; however, agonist-specific desensitization of the ASM β_2 AR is a prime and logical candidate. Not only would GRK- and arrestin-mediated desensitization of the β_2 AR be expected to occur as a consequence of chronic inhaled beta-agonist treatment, a recent study by Mak *et al.* (36) suggests that the homologous desensitization mechanism *per se* may be upregulated by the allergic inflammation of the airway. Given our current findings that demonstrate the relevance of arrestins in regulating ASM function and airway physiology, the dots appear to be connected to suggest that relief of β_2 AR desensitization by targeting arrestins has therapeutic potential. Drug delivery strategies that target arrestin expression or function could serve as a means of improving the efficacy of inhaled beta-agonist in bronchodilation, minimize lung inflammatory cell influx and its pathogenic effect (10), and thereby possibly thwart the loss of asthma control that occurs in the subset of asthmatics managed with chronic beta-agonists.

Acknowledgments

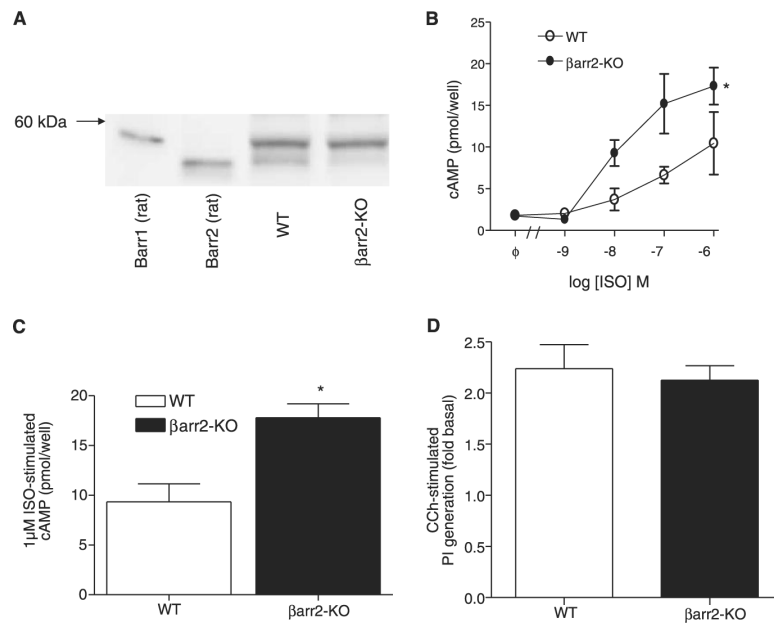
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**Figure 1.**

Effects of β arr-2 knockout on GPCR signaling in murine ASM cultures. *A*) Immunoblot analysis of β arr-1 and β arr-2 expression in mouse lung from WT and β arr-2-KO mice. Arrestin expression was detected using the A2CT Ab. β arr-2 knockout was confirmed by genotyping; faint reactivity below β arr-1 band in lysates from β arr-2-KO mice is nonspecific. Positive control lanes for β arr-1 and β arr-2 contained 2.5 and 0.25 ng of pure rat arrestins, respectively. *B*, *C*) ASM cultures derived from WT and β arr-2-KO mice were generated and cAMP accumulation stimulated by 10^{-9} – 10^{-6} ISO was assessed as described in Materials and Methods. *D*) CCh-stimulated phosphoinositide generation in ASM cultures derived from WT and β arr-2-KO mice. FSK-stimulated cAMP accumulation did not differ between groups (WT, 41 ± 2 pmol/well; β arr-2-KO, 44 ± 4 pmol/well). Values are means \pm SE from 3 (*B*) and 6 (*C*, *D*) experiments. * $P < 0.05$; 2-way ANOVA (*B*), *t* test for unpaired samples (*C*).

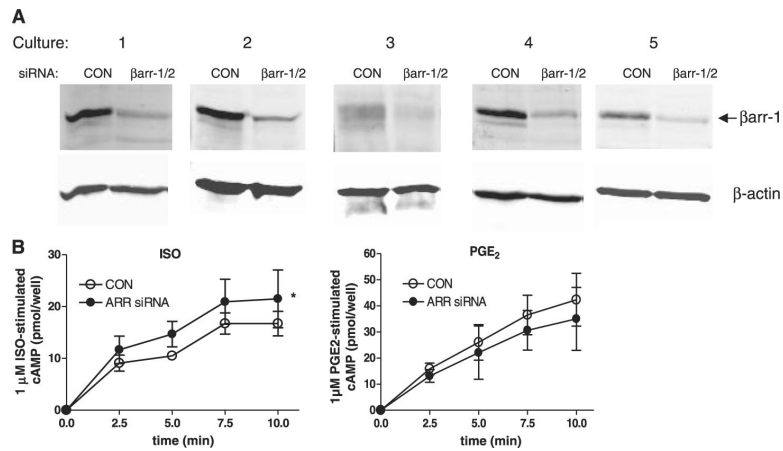
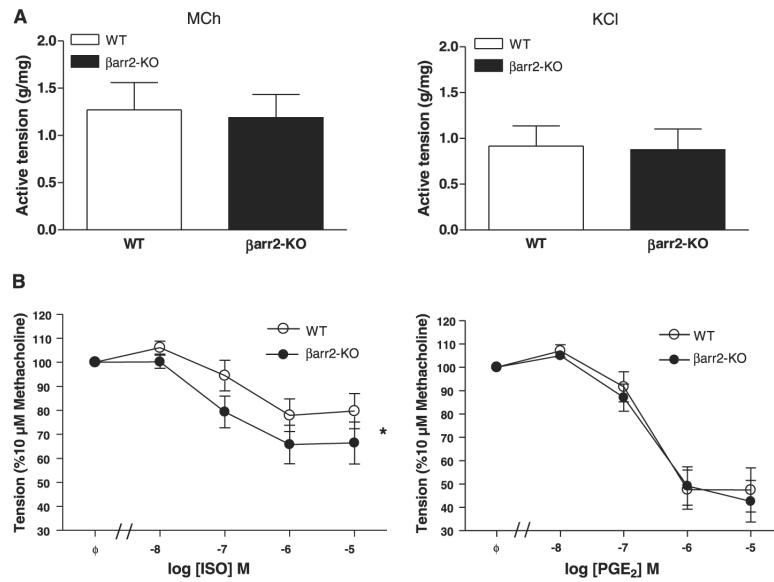


Figure 2.

Effects of β arr knockdown on GPCR signaling in human ASM cultures. *A*) Immunoblot analysis of arrestin expression in five distinct cultures transfected with scrambled (CON) or β arr-1/2 (ARR) siRNA oligonucleotides. *B*) Time-dependent cAMP accumulation in human ASM cultures stimulated with 1 μ M ISO (left panel) or 1 μ M PGE₂ (right panel). cAMP accumulation stimulated by 50 nM ISO (CON siRNA, 7.7 ± 1.5 pmol/well; ARR siRNA, 6.8 ± 1.1 pmol/well) or FSK (CON siRNA, 46 ± 5 pmol/well; ARR siRNA, 42 ± 4 pmol/well) did not differ between groups. Values represent mean \pm SE values from 5 experiments. * $P < 0.05$; ISO-stimulated cAMP accumulation CON vs. ARR siRNA, 2-way ANOVA.

**Figure 3.**

Effects of β arr-2 knockout on tracheal ASM contraction and relaxation *ex vivo*. Tracheal rings excised from WT and β arr-2-KO mice were mounted in a bath and contractile properties were assessed as described in Materials and Methods. *A*) Active tension development stimulated by 10 μ M MCh (left panel) or 60 mM KCl (right panel) was compared between WT and β arr-2-KO groups. *B*) The relaxant effect of increasing concentrations of ISO (left) or PGE₂ (right) on rings precontracted with 10 μ M MCh was compared between groups. FSK-mediated relaxation did not differ among groups ($78 \pm 7\%$ and $80 \pm 9\%$ reductions in WT and β arr-2-KO groups, respectively). Values are means \pm SE from 7 experiments. * $P < 0.05$; ISO effect on MCh-stimulated tension development WT and β arr-2-KO, 2-way ANOVA.

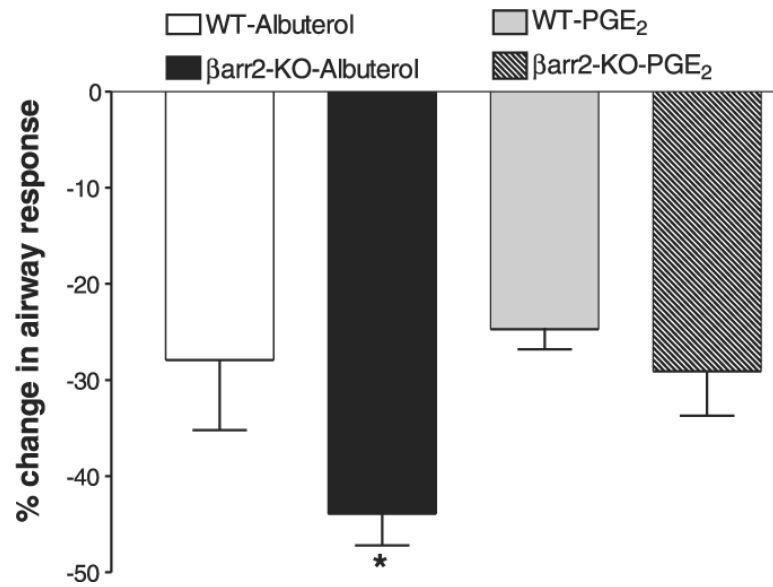


Figure 4.

Effects of β arr-2 knockout on airway responsiveness *in vivo*. To measure the effect of albuterol or PGE₂ on airway responsiveness, the magnitude of the APTI response to 100 μ g/kg MCh was compared to that of combined 100 μ g/kg MCh and 30 μ g/kg albuterol or 100 μ g/kg MCh and 5 μ g/kg PGE₂. The percentage change in airway response with albuterol administration is significantly greater in β arr-2-KO mice than in WT mice. Loss of β arr-2 does not alter bronchodilation in response to PGE₂ relative to WT mice. Values are means \pm SE from 6 to 8 mice per group. * P < 0.05; unpaired t test.

TABLE 1Effect of β arr-2 knockout on lung and ASM β -AR density

	WT ASM	βarr-2-KO ASM	WT lung	βarr-2-KO lung
β -AR density	80.3 \pm 25.2	88.9 \pm 13.8	444 \pm 56	471 \pm 38

Membranes from WT and β arr-2-KO mice were prepared as described in Materials and Methods. β -AR density was measured as radioligand binding (fmol/mg protein). Results are expressed as means \pm SD of 2 (lung) or 3 (ASM) experiments.