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RESEARCH ARTICLE

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Hyperinsulinemia promotes heterologous desensitization of β_2 adrenergic receptor in airway smooth muscle in obesity

Rui Xu[1,2] | Raghavender Reddy Gopireddy[3] | Yudi Wu[1] | Lei Wu[1] | Xiang Tao[1] | Ji Shao[1] | Wenxin Wang[1] | Li Li[1,2] | Aleksandra Jovanovic[3] | Bing Xu[3,4] | Nicolas J. Kenyon[5] | Quan Lu[6] | Yang K. Xiang[3,4] | Qin Fu[1,2]

[1]Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

[2]The Key Laboratory for Drug Target Researches and Pharmacodynamic Evaluation of Hubei Province, Wuhan, China

[3]Department of Pharmacology, University of California at Davis, Davis, CA, USA

[4]VA northern California Healthcare System, Mather, CA, USA

[5]Department of Medicine, University of California at Davis, Davis, CA, USA

[6]Department of Environmental Health, School of Public Health, Harvard University, Boston, MA, USA

Correspondence
Qin Fu, Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Email: fuqin@mails.tjmu.edu.cn

Yang K. Xiang, Department of Pharmacology, University of California at Davis, Davis, CA 95616, USA.
Email: ykxiang@ucdavis.edu

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Abstract

β-Adrenergic receptor (β-AR) agonists are the most common clinical bronchodilators for asthma. Obesity influences asthma severity and may impair response to β-AR agonists. Previous studies show that in obese mice, hyperinsulinemia plays a crucial role in β-AR desensitization in the heart. We therefore investigated whether insulin promotes β-AR desensitization in airway smooth muscle (ASM) and compromises airway relaxation responsiveness to β-AR agonists. We found that human ASM cells and mouse airway tissues exposed to insulin exhibit impaired β_2AR-induced cAMP accumulation and airway relaxation. This impaired relaxation is associated with insulin-induced phosphorylation and expression of phosphodiesterase 4D (PDE4D) through transactivation of a G protein-coupled receptor kinase 2 (GRK2)-dependent β_2AR-G_i-ERK1/2 cascade. Both acute and chronic pharmacological inhibition of PDE4 effectively reversed impaired β_2AR-mediated ASM relaxation in an obesity mouse model induced by a high fat diet. Collectively, these findings reveal that cross talk between insulin and β_2AR signaling promotes ASM β_2AR desensitization in obesity through upregulation of PDE4D phosphorylation and expression. Our results identify a novel pathway of asthma pathogenesis in patients with obesity/metabolic syndrome, in which the GRK2-mediated signaling can be a potential therapeutic modality to prevent or treat β_2AR desensitization in ASM. Moreover, PDE4 inhibitors may be used as efficacious therapeutic agents for asthma in obese and diabetic subjects.

Abbreviations: ASM, airway smooth muscle; β-AR, β-adrenergic receptor; ERK, extracellular regulated protein kinase; Fluo, fluoxetine; FRET, fluorescent resonance energy transfer; GRK2, G protein-coupled receptor kinase 2; HFD, high fat diet; IR, insulin receptor; ISO, isoproterenol; NC, normal chow; Paro, paroxetine; PDE4, phosphodiesterase 4; PGE2, prostaglandin E2; PKA, protein kinase A; PKC, protein kinase C; PTX, pertussis toxin; Roflu, Roflumilast; Roli, Rolipram.

Rui Xu, Raghavender Reddy Gopireddy and Yudi Wu are contributed equally to this study.

KEYWORDS
asthma, obesity, phosphodiesterase 4, β-adrenergic receptor agonist

1 | INTRODUCTION

Asthma is a common and potentially serious chronic airway disease. Obesity and diabetes could have a substantial role in the development, control, and severity of asthma.[1] Obese and diabetic subjects with asthma exhibit more severe or difficult-to-control clinical phenotypes; these patients display poor responses to inhaled β-adrenergic receptor (β-AR) agonists, which is a cornerstone in the treatment of asthma.[2]

Although the cause for this relationship between obesity/diabetes and asthma remains unclear, a number of relevant observations have shown the correlation between insulin and asthma abound. One of the hallmarks of diabetes is insulin resistance and associated hyperinsulinemia. Insulin resistance is a risk factor for asthma in children[3,4] and is also associated with developing asthma-like symptoms in adults.[5] Reports also indicate that high levels of insulin promote airway smooth muscle (ASM) contraction and enhance contractile responses to methacholine and KCl.[6,7] A recent study has shown that it is not obesity per se but hyperinsulinemia accompanying obesity that potentiates vagally induced bronchoconstriction by inhibiting neuronal M2 muscarinic receptors and increasing acetylcholine release from airway parasympathetic nerves.[8] Together, these results offer a potential explanation for the association between hyperinsulinemia and the severity of asthma. However, whether hyperinsulinemia leads to different cellular effects in the airway, and whether such effects can influence response to asthma medications such as β_2-agonists remains uncertain and should be elucidated.

β-adrenergic receptor agonists, acting on the β_2AR, are the most effective approach of bronchorelaxant therapy in airway diseases; however, the chronic use may lead to a decreased efficacy in bronchorelaxation and deterioration of asthma control,[9-11] which is thought to result from desensitization of the β_2AR.[12] Therapeutically, β-AR agonists acting on β_2ARs expressed on ASM cause bronchodilation via cAMP production. Phosphodiesterases (PDEs) act as key regulators of this pathway by degrading cyclic nucleotides. PDEs represent a super family of enzymes including 11 subfamilies that can degrade cytosolic cAMP and cGMP. In human ASM cells, PDE4 is the major PDE subfamily that is involved in cAMP degradation.[13] PDE4 plays a critical role in the control of airway smooth muscle contraction[14] and mediates airway hyperresponsiveness accompanying allergen challenge in asthmatic subjects.[15-17] Increased PDE4D expression promotes degradation of cAMP, leading to decreased β-AR responses in ASM from asthmatics.[18] Hu *et al*. also reported that prostaglandin E_2 (PGE_2)-induced heterologous β_2AR desensitization in ASM is mediated by upregulated phosphodiesterase (PDE4) expression.[19] Furthermore, it has been recently reported that roflumilast (Roflu), a PDE4 inhibitor, ameliorates airway hyperresponsiveness caused by diet-induced obesity in a murine model.[20]

Studies into the etiology of the homologous (agonist-specific) β_2AR desensitization in airways have provided valuable insights regarding the mechanisms involved in G protein-coupled receptor kinases (GRKs)-mediated phosphorylation of the β_2AR, which results in acute uncoupling of the β_2AR from its associated G_s protein-mediated accumulation of cAMP, leading to impaired ASM relaxation.[21,22] Meanwhile, a variety of elevated neurohormonal stimuli can impair heart contractile function via interaction with cardiac β-AR signal. This phenomenon is mainly attributed to heterologous desensitization of β-ARs, in which β-ARs are phosphorylated through activation of protein kinase A (PKA) or protein kinase C (PKC) in a β-AR agonist independent manner.[23,24] Recently, we have shown that both acute and chronic insulin stimulation induce heterologous β_2AR desensitization in the heart.[25,26] Hyperinsulinemia drives GRK phosphorylation of β_2AR, which compromises cardiac adrenergic stimulation via increases in coupling to G_i protein and increases in expression of PDE4D.[25,26] These studies suggest that hyperinsulinemia-related PDE4 upregulation can be a mechanism for increased β_2AR desensitization in ASM, parallel to those in the heart and contributes to the poor responsiveness to β-AR agonists in asthma patients with obesity. Therefore, the present study was taken to identify whether insulin induces heterologous β_2AR desensitization in ASM and the underlying mechanism.

2 | MATERIALS AND METHODS

2.1 | Animals care and use

The animal care and experimental protocols were approved by the institutional animal care and use committee of Tongji Medical College, Huazhong University of Science and Technology. Ten adult New Zealand white rabbits were used in this study of immunoblot analysis, and C57BL/6 mice were used in this study for ASM function assay in the normal and obesity model.

In brief, 5- to 6-week-old male C57BL/6J mice were randomly assigned to low fat or high fat diets (HFDs) for 5 months. After 4 months of HFD feeding, mice (n = 20) were randomly assigned to two groups. Each group was given daily oral gavage with vehicle or Roflu (1 mg/kg, Selleck Chemicals, Houston, TX, USA) for 1 month. The diets used for these studies were from HFK Bioscience Co. Ltd, (Beijing, China), the low fat was D12450B (10% kcal fat) and the HFD was D12492 (60% kcal fat diet). The mice had *ad libitum* access to food and they were housed in a room with a 12 hours light–12 hours dark cycle.

2.2 | Ex vivo analysis of relaxation responsiveness in ASM tissues

Murine trachea excised after euthanasia were cleaned of surrounding connective tissue and mounted into a multi-wire organ bath in modified Krebs-Ringer solution (pH 7.40-7.45) maintained at 37°C with 5% of CO_2 and 95% of O_2, with frequent changing of the solution. The airway segments were then attached to force transducers and multi-channel biological signal collecting/processing system (RM 6240BD, Chengdu Instrument, China) to monitor isometric tension. A basal tension of 0.5 *g* was set and the tracheal rings were allowed to equilibrate in the organ bath for 60 min. Their cholinergic contractility was assessed in response to cumulatively administration of acetylcholine (Ach) in final bath concentrations ranging from 10 nM to 100 μM. The rings were then rinsed three to four times over 15 minutes with fresh buffer, and the pharmacological agents were introduced into the organ bath. Each ring was incubated with either vehicle alone (control) or insulin (100 nM) for 0.5 hours, and in some experiments in the presence of either PDE4 inhibitor Roflu (0.5 nM), MEK1/2 inhibitor U0126 (1 μM), GRK2 inhibitor paroxetine (1 μM), serotonin reuptake inhibitor fluoxetine (1 μM), or G_i protein inhibitor pertussis toxin (PTX, 500 ng/mL). These agents were added to the organ bath 0.5 hours (except 3 hours for PTX) prior to the insulin pretreatment period. Thereafter, relaxations of dose-response curves to isoproterenol (ISO, 1 nM to 100 μM) were obtained after the tissues were treated with Ach (at ED_{50} doses) to generate half-maximally contraction. The relaxation dose-responses to ISO were obtained through normalizing isometric maximal relaxation response (Rmax) against the initial level of contraction induced by Ach.

2.3 | Cell culture and assay of cAMP activity

Human airway smooth muscle cells were cultured on glass coverslips in Ham's F-12 medium supplements with 10% of fetal bovine serum and maintained throughout in a humidified incubator containing 5% of CO2 in air at 37°C. In separate experiments, human ASM cells were cultured on glass coverslips and infected with regular ICUE3, an Epac based cAMP biosensor for 36 hours.[26] Coverslips with living ASM cells were treated with insulin 100 nM, for 0.5 hours or overnight in media and then maintained in PBS (KCl 2.68 mM, KH2PO4 1.47 mM, NaCl 136.89 mM, and Na2HPO4 8.1 mM) for fluorescent resonance energy transfer (FRET) recording as described previously.[26] Images were acquired using a Leica DMI3000 B inverted fluorescence microscope (Leica Biosystems, Buffalo Grove, IL) with a 40×/1.3 numerical aperture oil-immersion objective lens and a charge-coupled device camera controlled by Metafluor software (Molecular Devices, Sunnyvale, CA). FRET was recorded by exciting the donor fluorophore at 430-455 nm and measuring emission fluorescence with two filters (475DF40 for cyan and 535DF25 for yellow). The acquisition was set with 200-ms exposure in both channels and 20-s elapses. Images in both channels were subjected to background subtraction, and ratios of yellow-to-cyan color were calculated at different time points. The donor/acceptor FRET ratio was calculated and normalized to the ratio value of baseline. The binding of cAMP to ICUE3 decreased CFP–YFP FRET efficiency.

2.4 | Immunoblot analysis

Rabbits were killed after euthanasia with an intravenously administered overdose of 20% of ethyl carbamate (100 mg/kg) and the tracheae were excised, via open thoracotomy, the loose connective tissue and epithelium were scraped and removed. The tracheae were divided into twelve-ring segments, each of 4–6 mm in length, and then were placed in modified Krebs-Ringer solution and each alternate ring was incubated in the presence of either vehicle alone (control) or insulin (100 nM) for different time as indicated, both in the absence or presence of either Roflu (0.5 nM), U0126 (1 μM), paroxetine (1 μM) or pertussis toxin (500 ng/mL). These agents were added to the solution 0.5 hours (except 3 hours for PTX) prior to the insulin pretreatment period. Thereafter, the tissues lysates were prepared in lysis buffer (50 mM Tris, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 25 mM sodium pyrophosphate, and 1% (v/v) NonidetP40, 1% of Na-deoxycholate, 0.1% of SDS, and protease inhibitor cocktail tablets (Thermo Scientific, Chicago, IL) after washing twice with ice-cold PBS. The lysates without boiling were resolved by SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA), and membranes were probed with following antibodies: β_2AR (M-20, 1:400) and phospho-355 β_2AR (1:400) from SCBT (Santa Cruz, CA, USA), phospho-ERK (1:1000) and extracellular regulated protein kinase (ERK) (1:1000) from Cell Signaling (Danvers, MA, USA), PDE4D (1:400)

from Proteintech (Chicago, IL, USA), and phospho-PDE4D (S190) (1:400) from Abcam (Cambridge, UK). Chemiluminescent detection was performed with horseradish peroxidase–coupled secondary antibody from Cell Signaling (Danvers, MA, USA) and Super Signal West Femto reagent (Servicebio, Wuhan, China). Band densities were quantified using Image J software.

2.5 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA) and data are expressed as means ± SEM. Statistical analyses were performed using Student's two-tailed unpaired *t* test, one-way ANOVA followed by Tukey's multiple comparisons test or two-way repeated-measures ANOVA with Tukey's multiple comparisons test. A value of $P < .05$ was considered to indicate statistical significance. The sample size for each group is shown in the figure legends.

3 | RESULTS

3.1 | Insulin induces desensitization of β_2AR signaling in airway

We examined changes in intracellular cAMP following administration of the β-AR agonist ISO in human ASM cells pretreated for 30 min either with vehicle alone or with different concentrations of insulin. As shown in Figure 1A, the insulin-treated ASM cells exhibited reduced cAMP activity to ISO relative to those not exposed to insulin. Consequently, the relaxation responses to ISO were significantly attenuated in the insulin-treated ASM segments relative to control tissues (Figure 1B). The maximal relaxation response to ISO (10 μM) in the insulin-treated tissues amounted to 58.9 ± 2.9% *vs* the maximal relaxation response of 41.4 ± 6.4% obtained in the control segments ($P < .05$). We have recently showed that insulin-induced phosphorylation of β_2AR by GRKs is required for rapid receptor internalization and desensitization in cardiac myocytes. Thus, we hypothesized that insulin promotes phosphorylation of β_2AR, which attenuates receptor response to ISO, resulting lower accumulation of cAMP and smaller changes in ASM relaxation responsiveness. Indeed, insulin-induced β_2AR phosphorylation at the GRK site of serine 355/356 in time-dependent manner in rabbit ASM tissues (Figure 2A). Furthermore, paroxetine, a selective serotonin reuptake inhibitor that also has GRK2 inhibition activity rescued the relaxation responsiveness to ISO in insulin-treated ASM tissues (Figure 2B). Another selective serotonin reuptake inhibitor, fluoxetine that does not inhibit GRK2, did not affect insulin-mediated impaired relaxation response to ISO (EC_{50}: ISO, 0.35 μM; Ins + ISO, 10.3 μM; Paro + Ins + ISO, 0.61 μM; Fluo + Ins+ISO, 3.98 μM, Figure 2B). Accordingly, paroxetine, but not fluoxetine rescued ISO-induced cAMP activity in insulin-treated ASM cells (Figure 2C). In controls, paroxetine alone did not affect ISO-induced relaxation responsiveness (EC_{50}: ISO, 0.40 μM; Paro + ISO, 0.35 μM, Figure 2D) and cAMP activity (Figure 2E). Moreover, pretreatment with the G_i-protein inhibitor pertussis toxin (PTX) completely rescued ISO-induced cAMP activity in insulin-treated ASM cells (Figure 2F). These observations implicate that insulin drives phosphorylation of β_2AR to switch coupling from G_s to G_i protein and facilitates heterologous desensitization of β_2AR signaling in ASM.

Phosphodiesterase 4 plays an essential role in insulin-induced desensitization of β_2AR signaling in ASM. Phosphodiesterases promote desensitization of the β_2AR signaling via hydrolyzing cAMP. We examined whether insulin promotes desensitization of the β_2AR via increasing cAMP hydrolysis by PDEs in ASM. As depicted in Figure 3A, pretreatment with PDE4-selective inhibitor Roflu (0.5 nM) had no significant effect on relaxation responses to ISO in ASM tissues, but completely rescued the impaired relaxation response to ISO detected in insulin-treated ASM tissue (EC_{50}: ISO, 0.62 μM; Ins+ISO, 13.0 μM; Roflu + ISO, 0.70 μM; Roflu + Ins + ISO, 0.37 μM). Consistent with the relaxation responses, pretreatment with the selective PDE4 inhibitor rolipram (Roli), completely rescued the ISO-induced cAMP activity in ASM cells exposed to insulin. In comparison, a selective PDE3 inhibitor cilostamide failed to do so (Figure 3B). In controls, neither PDE inhibitor affected cAMP activity of ASM cells at baseline, nor did they affect the cAMP levels induced by ISO in the absence of insulin (Figure 3C).

Among PDE4 isoforms, PDE4D functionally associated with β_2AR to fine-tune receptor-induced cAMP activity.[27] The enzymatic activity is modulated at the expression level and through phosphorylation-dependent activation. ASM tissues exposed to insulin exhibited time-dependent increases in phosphorylation of PDE4D (Figure 3D). The increased phosphorylation and expression of PDE4D was ablated by Roflu (Figure 3E). These observations implicate that activation of PDE4D contributes to the impaired β_2AR-induced cAMP activity following insulin-induced heterologous desensitization of receptor signaling in ASM cells.

3.2 | Insulin induces ERK1/2-dependent activation of PDE4D in ASM tissues

Recent reports implicate a critical role of ERK1/2 activation in mediating the hyporesponsiveness of ASM to β_2AR stimulation under different proasthmatic conditions.[28] Our recent study demonstrated that insulin promotes PDE4D expression by a GRK2-dependent transactivation of a

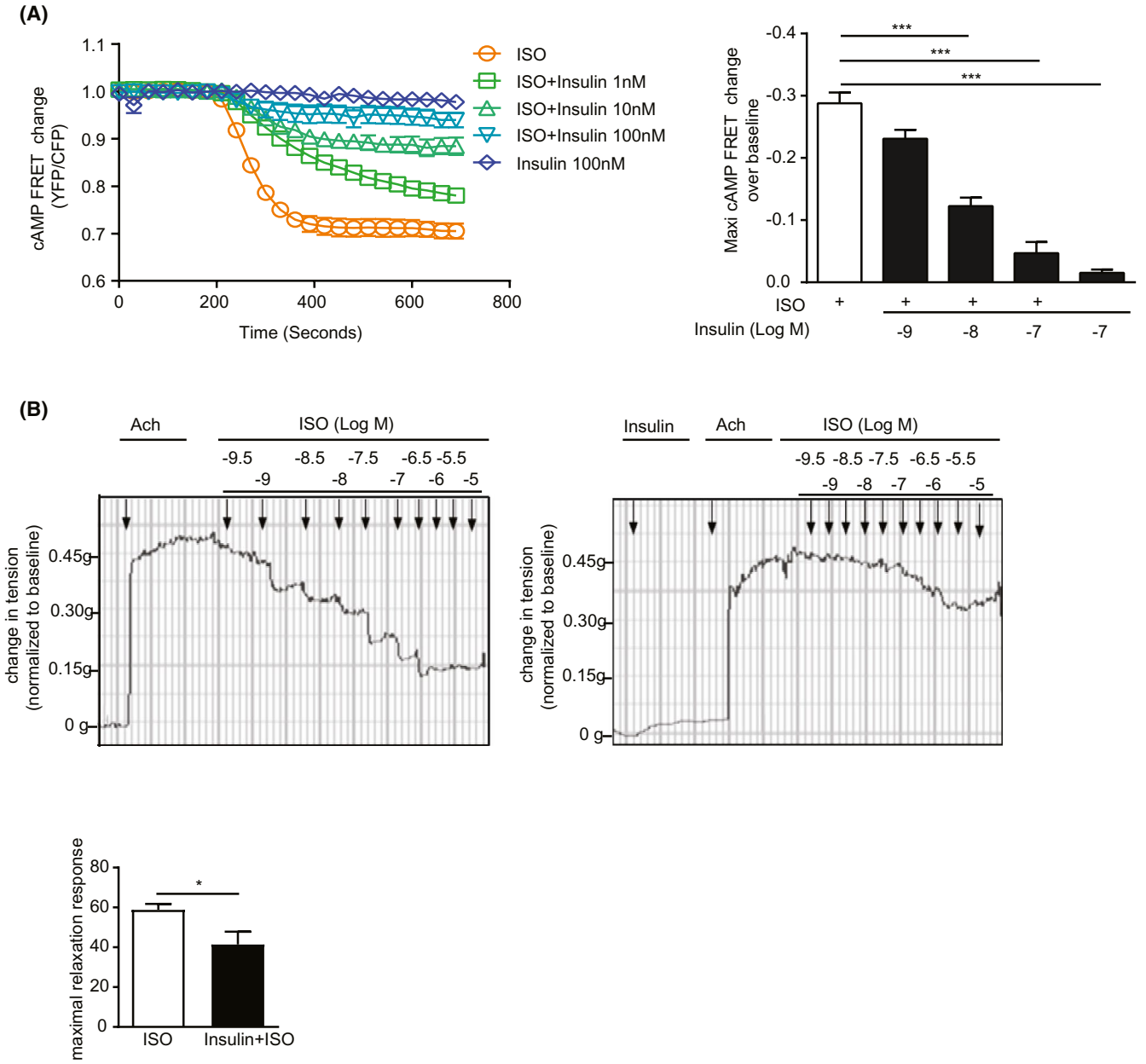

FIGURE 1 Insulin induces heterologous β_2AR desensitization in cultured human airway smooth muscle cells and mouse airway smooth muscle. A, Human ASM cells (HASMC) expressing cAMP biosensor ICUE3 were stimulated with ISO in the absence or presence of insulin at different concentrations as indicated. The traces show time courses of changes in FRET ratio and the maximal increases in FRET ratio were plotted. Values are means ± SEM (n = 17-21). B, Tracheal rings excised from mice were stimulated with acetylcholine (Ach) followed by ISO at different concentrations in the absence or presence of insulin (100 nM, 0.5 hours). Data show a representative curve of relaxant response to ISO. The maximal relaxation responses were plotted (n = 7). ***$P < .001$ by one-way ANOVA followed by a Tukey's multiple comparisons test

β_2AR-ERK1/2 signaling cascade in heart.[25] A GRK2 inhibitor paroxetine prevented the increases in phosphorylation of β_2AR and PDE4D in insulin-treated ASM (Figure 4A). ASM tissues exposed to insulin also exhibited increased expression of phosphorylated ERK1/2, with peak phosphorylation detected at 30 min (Figure 4B). Inhibition of ERK1/2 with U0126 prevented the insulin-induced phosphorylation and expression of PDE4D in ASM tissues (Figure 4C). Accordingly, pretreatment with ERK1/2 inhibitor U0126 rescued the relaxation response to ISO in insulin-treated ASM tissues (EC_{50}: ISO, 0.62 μM; Ins + ISO, 13.0 μM; U0126+ISO, 1.1 μM; U0126 + Ins + ISO, 2.53 μM, Figure 4D). Meanwhile, GRK2 inhibitor paroxetine blocked insulin-induced ERK1/2 phosphorylation in ASM tissues (Figure 4E). Together, these observations implicate that insulin promotes a GRK2-mediated transactivation of the β_2AR-ERK1/2 signaling cascade in ASM, which promotes PDE4D phosphorylation and expression.

Furthermore, pretreatment with the G_i inhibitor, PTX, prevented insulin-induced increases in ERK1/2 phosphorylation in ASM tissues (Figure 4F), consistent with the report that β_2AR-G_i coupling mediates activation of ERK1/2 signaling in β_2AR agonist-exposed ASM.[28] As expected, PTX also ablated the insulin-induced PDE4D phosphorylation and expression in the ASM (Figure 4G). Taken together, these data support the concept that insulin promotes heterologous

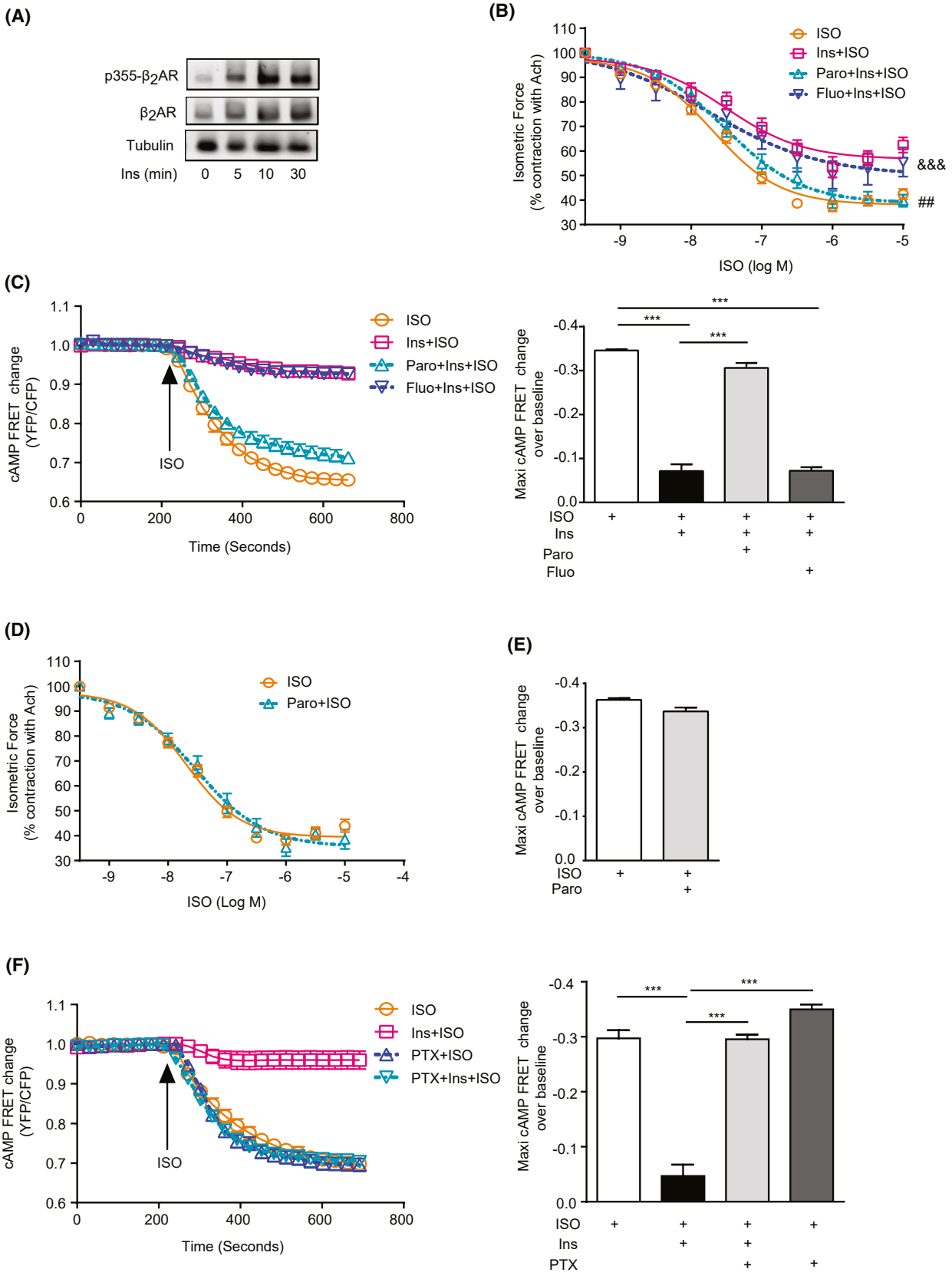

FIGURE 2 Inhibition of GRK2 and G_i prevents insulin-mediated β_2AR desensitization. A, Rabbit tracheal rings were stimulated with 100 nM insulin for indicated times and the levels of phosphorylation of β_2AR at 355/356 were detected by western blot (n = 3). B, The insulin (100 nM, 0.5 hours) induced changes in ASM relaxation responsiveness were abrogated in tissues that were pretreated with paroxetine (1 μM, 0.5 hours) but not fluoxetine (1 μM, 0.5 hours). Values are means ± SEM (n = 8-10). C, HASMC expressing cAMP biosensor ICUE3 were pretreated with insulin (100 nM, 0.5 hours) in the absence or presence of paroxetine (1 μM, 0.5 hours) or fluoxetine (1 μM, 0.5 hours) before stimulation with ISO and the changes in cAMP FRET ratio were recorded (n = 20-24). D, Relaxant effect of increasing concentrations of ISO on tracheal rings precontracted with Ach in the presence or absence of paroxetine (1 μM, 0.5 hours). Values are means ± SEM (n = 6-8). E) HASMC expressing cAMP biosensor ICUE3 were stimulated with ISO in the absence or presence of paroxetine (1 μM, 0.5 hours) and the changes in cAMP FRET ratio were recorded (n = 20-24). F, Human ASM cells expressing cAMP biosensor ICUE3 were pretreated with insulin in the absence or presence of pertussis toxin (500 ng/mL, 3 hours) before stimulation with ISO and the changes in cAMP FRET ratio were recorded (n = 20-29). ***$P < .001$, one-way ANOVA followed by a Tukey's test; $^{\&\&\&}P < .001$ vs ISO, ## $P < .01$ *vs* Insulin + ISO, two-way ANOVA followed by a Tukey's multiple comparisons test

β_2AR desensitization by upregulation of PDE4 activity via a GRK2-mediated transactivation of a β_2AR-G_i-ERK1/2 signaling cascade in ASM tissues.

3.3 | Chronic insulin stimulation induces desensitization of β_2AR signaling in airway

Next, ASM tissues were exposed to insulin overnight to mimic the hyperinsulinemia in obesity. PDE4 inhibitor Roli and Roflu rescued the ISO-induced cAMP activity in ASM cells exposed to insulin overnight (Figure 5A). Accordingly, the phosphorylation and expression of PDE4D were also upregulated by chronic insulin stimulation, which were prevented by PDE4 inhibitor Roflu (Figure 5B), GRK2 inhibitor paroxetine (Figure 5C), G_i protein inhibitor PTX (Figure 5D), and ERK1/2 inhibitor U0126 (Figure 5E).

3.4 | Inhibition of PDE4 ameliorates β_2AR desensitization in ASM in a murine model of obesity

We then examined the effects of high levels of insulin associated with feeding a high fat diet (HFD) on the relaxation response to ISO in ASM. We fed C57BL/6J mice with 60% HFD for 5 months, which resulted in significant body weight gain and elevated fasting glucose and insulin levels (data not shown). As expected, ASM from HFD-fed mice displayed reduced relaxation responses to ISO, which was ameliorated by acute Roflu treatment (EC50: NC, 0.46 μM; NC + Roflu, 0.37 μM; HFD, 2.13 μM; HFD+Roflu, 0.68 μM, Figure 6A). Meanwhile, the phosphorylation of Akt in ASM from HFD mice treated with insulin was impaired, which indicated the existence of insulin resistance in HFD mice (Figure 6B). Given that human studies have reported the improvement in insulin resistance after treatment with PDE4 inhibitor Roflu,[29] we evaluated the chronic effects of PDE4 inhibitor Roflu. The mice were subjected to 1-month therapy with Roflu after 4 months HFD feeding. Chronic Roflu treatment significantly improved relaxant response to ISO in ASM (Figure 6C) as well as the local insulin resistance in ASM (Figure 6D) and systemic insulin resistance (data not shown)

4 | DISCUSSION

Inhaled selective β_2AR agonists are the most widely used treatment for acute relief of asthmatic symptoms. Recent studies have attributed the development of airway tolerance to chronic use of β-AR agonists to homologous β_2AR desensitization of the ASM. Similarly, obese patients may display poor responsiveness to β-AR agonists. In the present study, we provide evidence demonstrating that (i) Acute or chronic exposure ASM to insulin leads to heterologous β_2AR desensitization accompanied with the impaired relaxation responsiveness; (ii) this desensitization is attributed to insulin-induced and GRK2-mediated transactivation of β_2AR-G_i-ERK1/2 pathway, which leads to upregulation of PDE4D phosphorylation and expression. Activated PDE4D enhances degradation of cAMP, which contributes to decreased relaxation responsiveness to β-AR agonist in ASM; and (iii) Acute or chronic pharmacological inhibition of PDE4 can effectively reverse β_2AR desensitization in ASM in mice with HFD-induced obesity.

Hyperinsulinemia is a hallmark of type 2 diabetes. While it is widely regarded as a compensatory adaptation due to systemic insulin resistance associated impaired glucose uptake. Insulin is generally considered necessary in diabetic patients to improve glycemic control. However, recent studies indicate that aggressive hyperglycemic controls may be associated with worsening cardiovascular outcomes.[30] We demonstrated that insulin-induced β_2AR/Gi coupling is sufficient to attenuate adrenergic-induced cAMP activities in hearts.[26] Our recent study indicates that hyperinsulinemia can be detrimental by driving down cardiac adrenergic signaling transduction via PDE4, contributing diabetic cardiomyopathy.[25] PDE4 has been characterized as a key

regulator to promote homologous desensitization of β_2AR in fibroblasts and cardiac myocytes. Subsequently, these enzymes have been shown to be physically associated with the β_2AR for tight control of cAMP-PKA activity induced by receptor activation. More recently, these enzymes are implicated in heterologous desensitization of β_2AR in cardiac myocytes by a variety of neurotransmitters and hormones including insulin[31] and prostaglandin E2.[32]

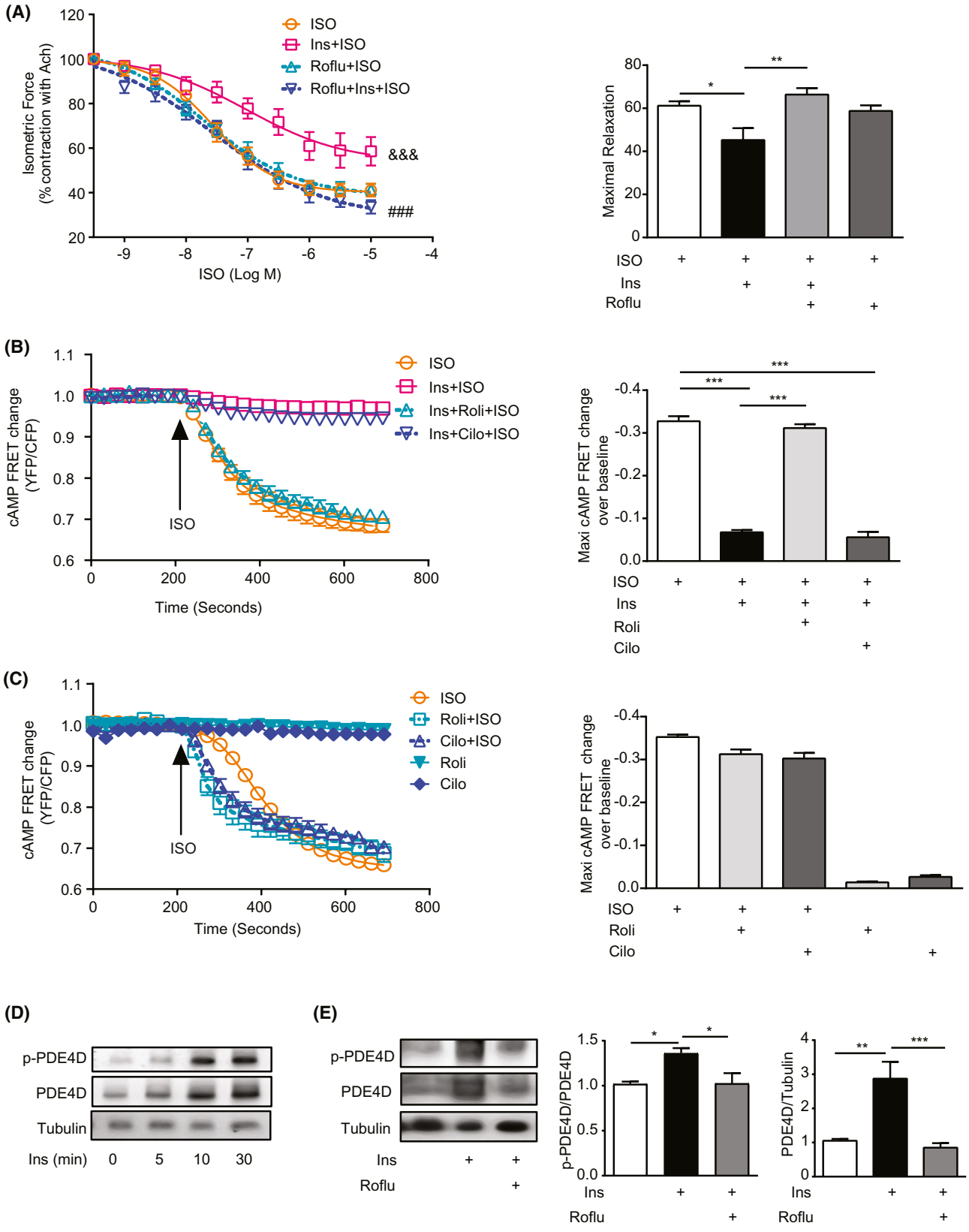

FIGURE 3 Inhibition of PDE4 prevents insulin-mediated β_2AR desensitization. A, Pretreatment with the PDE4 inhibitor roflumilast (0.5 nM, 0.5 hours) prevented the insulin-induced changes in ASM relaxation responsiveness while having no effect on ASM responsiveness in vehicle-exposed tissues. The maximal relaxation responses were plotted (n = 6-9). B) HASMC expressing cAMP biosensor ICUE3 were pretreated with insulin (100 nM, 0.5 hours) in the absence or presence of the PDE4 inhibitor rolipram (100 nM, 0.5 hours) or PDE3 inhibitor cilostamide (1 μM, 0.5 hours) before stimulation with ISO and the changes in cAMP FRET ratio were recorded (n = 24-28). C, Human ASM cells expressing cAMP biosensor ICUE3 were stimulated with ISO in the absence or presence of rolipram (100 nM, 0.5 hours) or cilostamide (1 μM, 0.5 hours). The changes in cAMP FRET ratio were recorded (n = 17-18). D) Rabbit tracheal rings were stimulated with 100 nM insulin for indicated times and the levels of phosphorylation and expression of PDE4D were detected by western blot (n = 3). E, The rings were stimulated with 100 nM insulin for 0.5 hours in the presence or absence of roflumilast (0.5 nM, 0.5 hours). Phospho-PDE4D and total PDE4D were then detected by western blot (n = 4). PDE4D phosphorylation was normalized against total PDE4D. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, one-way ANOVA followed by a Tukey's test. $^{\&\&\&}P < .001$ vs ISO, $\#\#\#P < .01$ vs Insulin + ISO, two-way ANOVA followed by a Tukey's multiple comparisons test

Here, we observed that insulin promotes desensitization of β_2AR via evoking phosphorylation of β_2AR at the GRK site p355/356, which transduces G_i-dependent ERK activation to promote phosphorylation and expression of PDE4D in ASM. Consequently, PDE4 promotes cAMP hydrolysis, leading to the impaired ASM relaxant responsiveness to β_2AR agonist ISO; and inhibition of PDE4 prevents the impaired acute ISO-induced accumulation of cAMP in human ASM cells exposed to insulin (Figure 3B). Blocking insulin-induced transactivation of β_2AR with GRK2 inhibitor abolishes insulin-induced ERK activation, PDE4D phosphorylation and expression (Figure 4A,E), and the inhibitory effect of insulin on relaxation response to ISO (Figure 2B). Inhibition of ERK1/2 also resulted in similar effects (Figure 4D).

Studies in Houslay's lab showed initially that ERK1/2 activation causes a direct inhibitory phosphorylation action of PDE4D5 in smooth muscle cells,[33] which is in contrast to our study that ERK1/2 stimulate PDE4 activity in airway smooth muscle tissue. Subsequently, they reported that ERK1/2 activation also activates an autocrine pathway that allows PKA to rapidly phosphorylate PDE4D5. As activation of cAMP-dependent PKA causes an increase in cAMP degradation via phosphorylation and activation of PDE4,[34] this feedback effect on PKA reprograms ERK from causing net inhibition to net activation of PDE4D5.[33] Moreover, a recent study notes that the cultured human ASM cells derived from asthmatic donor lungs exhibit constitutively increased PDE activity that is attributed to intrinsically upregulated Gβγ signaling to c-Src-dependent activation of the Ras/MEK/ERK1/2 cascade. The heightened baseline PDE activity in asthmatic human ASM cells is reduced to near normal levels following 2 hours of treatment with MEK-ERK1/2 inhibitor, U0126.[19] These results agreed with the present observation that treatment with MEK-ERK1/2 inhibitor U0126 suppressed the PKA phosphorylation of Ser190 of PDE4D in ASM tissues exposed to insulin. Together, these findings support a notion that hyperinsulinemia may exacerbate asthma by promoting heterologous β_2AR desensitization in ASM, which is mediated by increased PDE4 activity via transactivation of ERK1/2.

The current study also highlights a critical role of PDE4D in desensitization of β_2AR in ASMs from obesity mice. Our results confirmed that HFD mice had decreased relaxation responses in ASM to ISO, and acute treatment with PDE4 inhibitor Roflu rescued the relaxant response. Moreover, 1-month therapy with Roflu significantly ameliorated HFD-associated hyperglycemia, hyperinsulinemia, and systemic insulin resistance (data not shown) as well as the local insulin resistance and relaxant response in ASM indicating that improvement in insulin resistance could also contribute, in part, to the beneficial effects of PDE4 inhibition on relaxation responsiveness in ASM. Other experimental models of lung diseases have shown that Roflu significantly attenuates lung fibrosis, ROS production, and adipokine and cytokine levels. These factors may also contribute to the amelioration of airway hyperresponsiveness during chronic therapy with Roflu.[20] Another intriguing beneficial side effect clinically, is that Roflu may also provide relief of obesity-associated asthma by inducing weight loss.[20] Together, these findings indicate that Roflu may be an effective drug for the treatment of obesity-associated asthma.

In this study, the mechanisms through which hyperinsulinemia induce β_2AR desensitization remain to further explored. Whether the insulin receptor (IR) and/or its substrates are involved in the cross talk with β_2AR remain unclear. Studies in cardiac tissues show that the IR forms a complex with β_2AR, and activation of IRs leads to IRS-dependent recruitment of GRK2 to the complex,[26] which promotes phosphorylation of β_2AR. Although the role of IR proteins and GRK2 were not thoroughly investigated, GRK2 inhibitor paroxetine significantly attenuated ERK activity and PDE4D phosphorylation, indicating a conserved mechanism underlying the cross talk between IR and β_2AR in ASMs. Thus, further studies using GRK2 inhibitor and GRK2 knockout mice will be helpful to understand the role of GRK2 in HFD-induced β_2AR desensitization in airway smooth muscle models. In addition, the recruitment β-arrestin to GRK-phosphorylated β_2AR is a major determinant of both homologous and heterologous desensitization of the receptor. Indeed, a recent study has indicated β-arrestin-2 specifically desensitizes β_2AR and constrains β-AR agonist induced signaling and relaxation in ASM.[35]

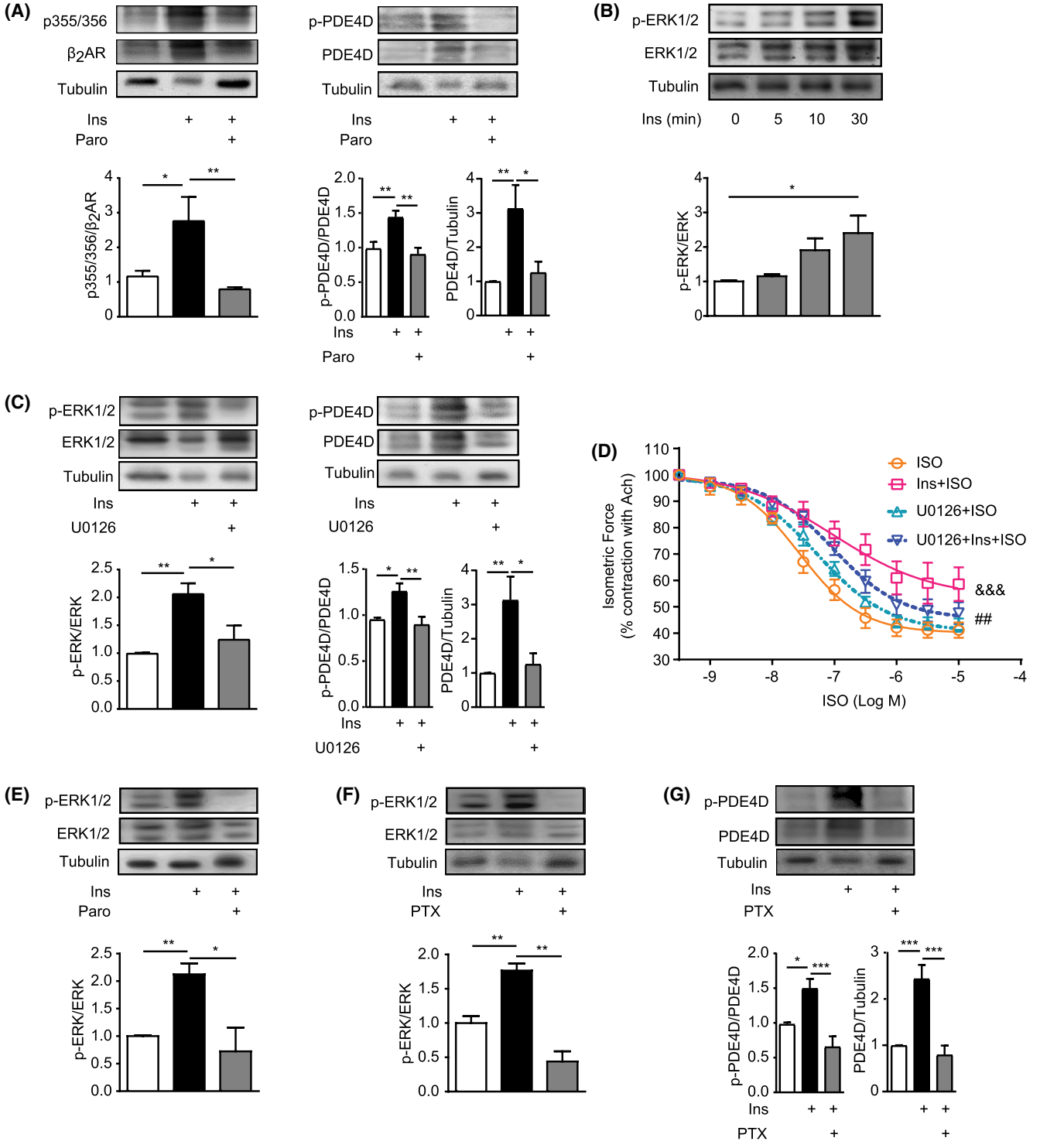

FIGURE 4 Inhibition of ERK1/2 rescues insulin-mediated β_2AR desensitization. A, Tracheal rings were stimulated with 100 nM insulin for 0.5 hours in the presence or absence of paroxetine (1 μM, 0.5 hours). Phosphorylation of β_2AR at 355/356 and PDE4D were detected by western blot (n = 4). B) Tracheal rings were stimulated with 100 nM insulin for the indicated times, and the levels of phosphorylation of ERK1/2 were detected by western blot and normalized against total ERK1/2 (n = 4). C, The rings were pretreated with U0126 (1 μM, 0.5 hours) before stimulation with 100 nM insulin for 0.5 hours. Phosphorylation of ERK1/2 and PDE4D were detected by western blot (n = 4). D, The insulin (100 nM, 0.5 hours) induced changes in ASM relaxation responsiveness were rescued in tissues pretreated with ERK1/2 inhibitor U0126 (1 μM, 0.5 hours) (n = 6-8). E-G, The rabbit trachea rings were pretreated with either GRK2 inhibitor paroxetine (1 μM, 0.5 hours) or G_i inhibitor PTX (500 ng/mL, 3 hours) before stimulation with 100 nM insulin for 0.5 hours. Phosphorylation of ERK1/2 and PDE4D were then detected by western blot (n = 4). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, one-way ANOVA followed by a Tukey's test; $^{\&\&\&}P < .001$ vs ISO, $^{\#\#}P < .01$ vs Insulin + ISO, two-way ANOVA followed by a Tukey's multiple comparisons test

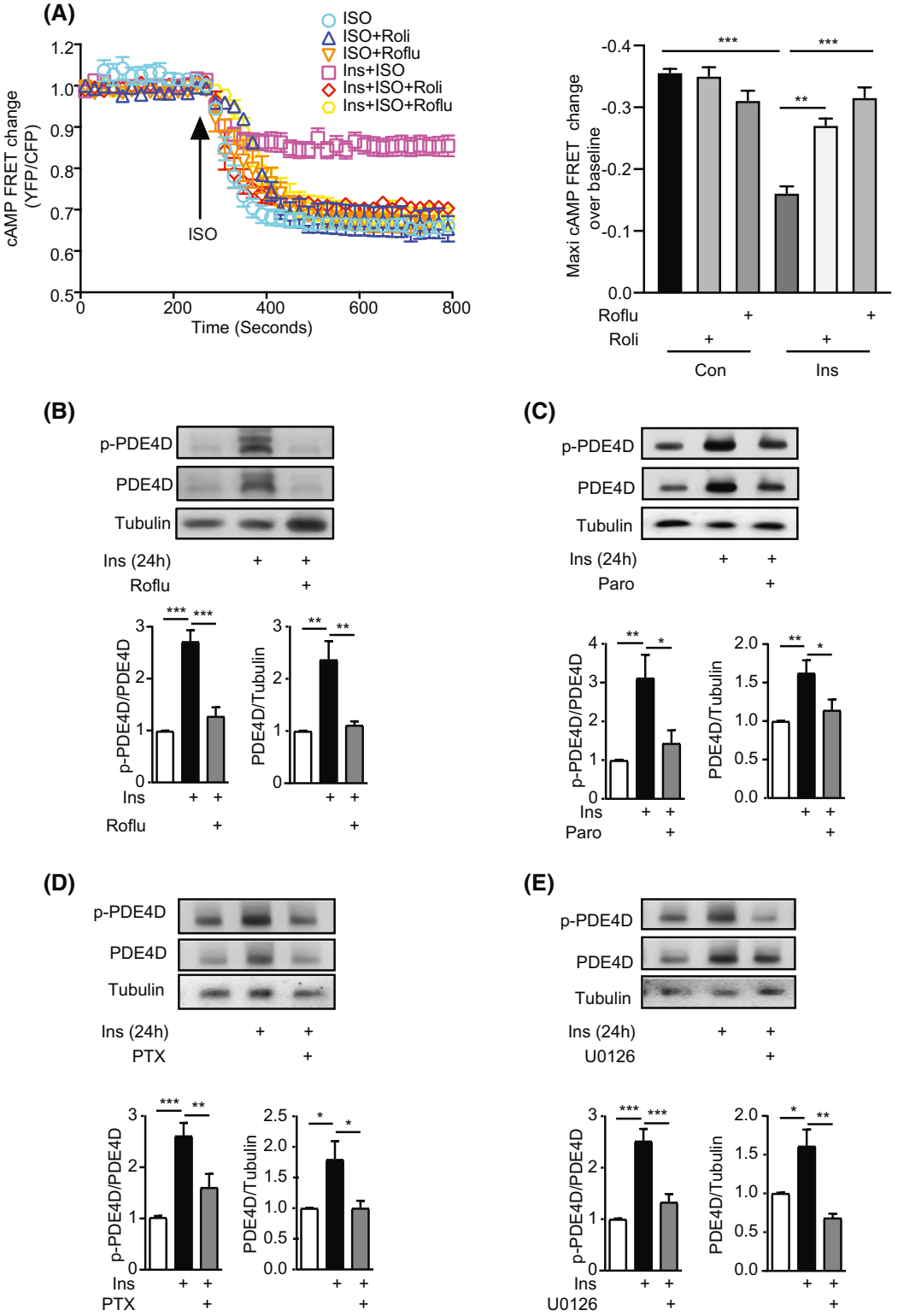

FIGURE 5 Inhibition of PDE4 prevents chronic insulin stimulation-mediated β_2AR desensitization. A, HASMC expressing cAMP biosensor ICUE3 were pretreated with insulin (100 nM, overnight) in the absence or presence of the PDE4 inhibitor rolipram (100 nM, overnight) or roflumilast (0.5 nM, overnight) before stimulation with ISO and the changes in cAMP FRET ratio were recorded (n = 16-28). B, Rabbit tracheal rings were stimulated with 100 nM insulin for 24 hours in the presence or absence of roflumilast (0.5 nM, 0.5 hours) and the levels of phosphorylation and expression of PDE4D were detected by western blot (n = 3). C-E, The rings were stimulated with 100 nM insulin for 24 hours in the presence or absence of paroxetine (1 μM, 0.5 hours), G_i inhibitor PTX (500 ng/mL, 3 hours) or ERK1/2 inhibitor U0126 (1 μM, 0.5 hours). Phospho-PDE4D and total PDE4D were then detected by western blot (n = 4). PDE4D phosphorylation was normalized against total PDE4D. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, one-way ANOVA followed by a Tukey's test

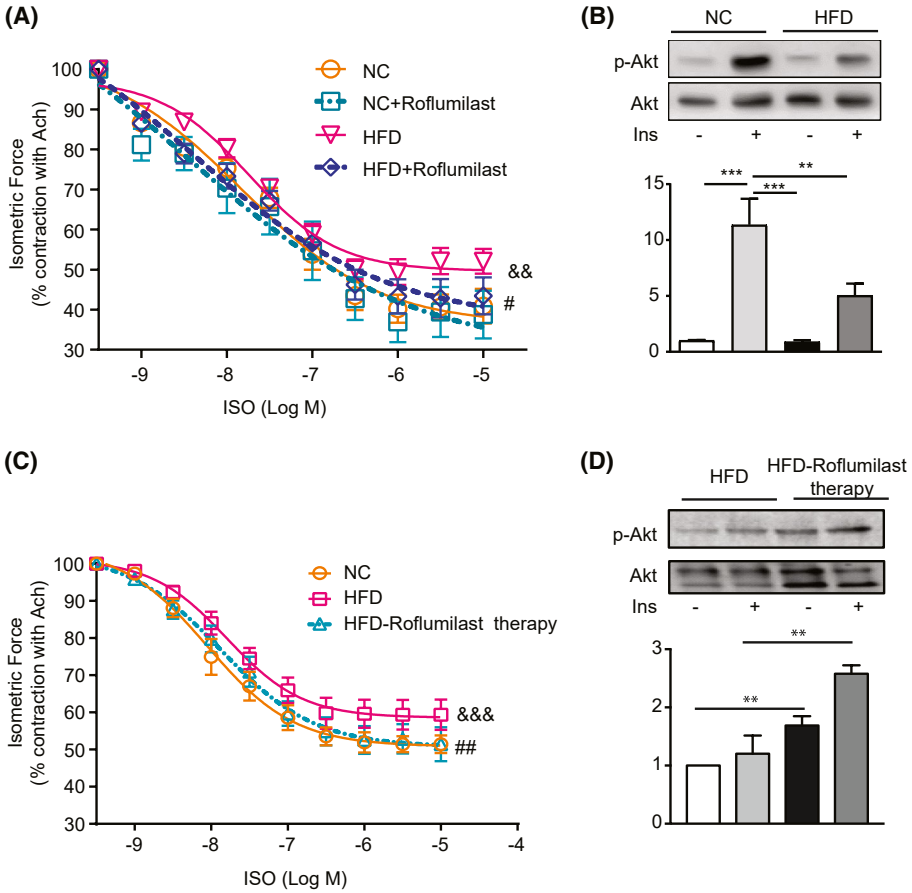

FIGURE 6 PDE4 inhibition restores the relaxant response to β-AR agonist and insulin signaling in ASM of HFD mice. A, HFD led to decreased relaxation response to ISO in mouse ASM. Roflumilast (0.5 nM, 0.5 hours) rescued ASM relaxation response in HFD model (n = 6). B, HFD led to reduction of insulin-induced Akt phosphorylation in ASM. C-D, Roflumilast treatment (1 mg/kg, i.p) for 1 month rescued ASM relaxation response (n = 8-9, C, and insulin-induced Akt phosphorylation (D) in HFD model. $^{\&\&\&}P < .001$ vs NC, $^{\#\#}P < .01$ vs HFD, two-way ANOVA followed by a Tukey's multiple comparisons test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, one-way ANOVA followed by a Tukeys test

Therefore, the role of β-arrestin-2 in insulin-induced β_2AR desensitization needs to be further investigated.

Recent advances in asthma therapy have concentrated on the treatment of allergic inflammation and its role in the pathogenesis of asthma. However, while obesity and diabetes-related asthma is an increasingly recognized entity, there are no specific therapies for these unique patients. Our results suggest that Roflu may be used as a novel therapeutic agent against asthma associated with obesity or diabetes. In addition, the novel use of the commonly used GRK2 inhibitor paroxetine can also be a potential therapeutic modality to prevent or treat β_2AR desensitization in ASM associated with hyperinsulinemia. Interventions targeted at regulatory sites within this insulin-β_2AR cross talk network may provide novel therapeutic approaches to ameliorate the poor responses to β-AR agonists in the treatment of asthma in obesity/diabetic patients.

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CONFLICT OF INTERESTS

Authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Q. Fu and Y.K. Xiang designed research; R. Xu, R.R. Gopireddy, and Y. Wu analyzed data; R. Xu, R.R. Gopireddy, Y. Wu, L. Wu, X. Tao, J. Shao, W. Wang, L. Li, A. Jovanovic, and B. Xu performed research; Q. Fu, N.J. Kenyon, Q. Lu, and Y.K. Xiang wrote the paper and Q. Lu contributed human airway smooth muscle cells.

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