

Long-Acting β_2 -Adrenoceptor Agonists Enhance Glucocorticoid Receptor (GR)-Mediated Transcription by Gene-Specific Mechanisms Rather Than Generic Effects via GR[Ⓢ]

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ABSTRACT

In asthma, the clinical efficacy of inhaled corticosteroids (ICSs) is enhanced by long-acting β_2 -adrenoceptor agonists (LABAs). ICSs, or more accurately, glucocorticoids, promote therapeutically relevant changes in gene expression, and, in primary human bronchial epithelial cells (pHBECs) and airway smooth muscle cells, this genomic effect can be enhanced by a LABA. Modeling this interaction in human bronchial airway epithelial BEAS-2B cells transfected with a 2× glucocorticoid response element (2×GRE)-driven luciferase reporter showed glucocorticoid-induced transcription to be enhanced 2- to 3-fold by LABA. This glucocorticoid receptor (GR; NR3C1)-dependent effect occurred rapidly, was insensitive to protein synthesis inhibition, and was maximal when glucocorticoid and LABA were added concurrently. The ability of LABA to enhance GR-mediated transcription was not associated with changes in GR expression, serine (Ser²⁰³, Ser²¹¹, Ser²²⁶) phosphorylation, ligand affinity,

or nuclear translocation. Chromatin immunoprecipitation demonstrated that glucocorticoid-induced recruitment of GR to the integrated 2×GRE reporter and multiple gene loci, whose mRNAs were unaffected or enhanced by LABA, was also unchanged by LABA. Transcriptomic analysis revealed glucocorticoid-induced mRNAs were variably enhanced, unaffected, or repressed by LABA. Thus, events leading to GR binding at target genes are not the primary explanation for how LABAs modulate GR-mediated transcription. As many glucocorticoid-induced genes are independently induced by LABA, gene-specific control by GR- and LABA-activated transcription factors may explain these observations. Because LABAs promote similar effects in pHBECs, therapeutic relevance is likely. These data illustrate the need to understand gene function(s), and the mechanisms leading to gene-specific induction, if existing ICS/LABA combination therapies are to be improved.

Introduction

Known as inhaled corticosteroids (ICSs), synthetic glucocorticoids act on the glucocorticoid receptor (GR; gene symbol NR3C1) to reduce inflammatory gene expression and are

generally effective in controlling mild-to-moderate asthma (Barnes, 2011; Oakley and Cidlowski, 2013). However, in a subset of severe asthmatics, ICSs provide insufficient control, and add-on therapies are recommended (Reddel et al., 2015; Newton and Giembycz, 2016). Probably the most widely prescribed option are long-acting β_2 -adrenoceptor agonists (LABAs), which are administered in combination with ICS, to improve lung function, reduce exacerbation frequency, and improve quality of life, more than increasing the ICS dose (Newton and Giembycz, 2016). Such data suggest that ICSs and LABAs interact at a molecular level to improve therapeutic outcomes (Giembycz et al., 2008). Several mechanisms have been advanced to explain this effect, including the ability of LABAs to enhance, in a cAMP-dependent manner, glucocorticoid-driven

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ABBREVIATIONS: Genes, mRNAs, or proteins, unless otherwise indicated, are referred to by the official gene symbol, as supplied by the National Center for Biotechnology Information. ChIP, chromatin immunoprecipitation; DAPI, 4',6-diamidino-2-phenylindole; Dex-Mes, dexamethasone 21-mesylate; GBS, GR binding site; GR, glucocorticoid receptor; GRE, glucocorticoid response element; ICS, inhaled corticosteroid; LABA, long-acting β_2 -adrenoceptor agonist; PCR, polymerase chain reaction; pHBEC, primary human bronchial epithelial cell; PKA, protein kinase A; qPCR, quantitative real-time PCR; siRNA, small interfering RNA.

transcription from a simple glucocorticoid response element (GRE)-dependent reporter (Kaur et al., 2008). In this simple system, LABAs alone are without effect, yet synergistically increase the maximal glucocorticoid-induced response. This mimics the clinical situation in which LABAs are steroid-sparing (Newton and Giembycz, 2016).

Mechanistically, LABAs have been suggested to promote translocation of GR to the nucleus (Roth et al., 2002; Usmani et al., 2005; Mortaz et al., 2008; Haque et al., 2013), even independently of a glucocorticoid (Eickelberg et al., 1999). Alternatively, the cAMP pathway may increase GR expression and agonist affinity and/or increase GR DNA binding (Dong et al., 1989; Rangarajan et al., 1992; Korn et al., 1998). However, other studies do not corroborate these effects (Gruol and Altschmied, 1993; Moyer et al., 1993; Zhang et al., 1993; Lovén et al., 2007). Nevertheless, expression of many glucocorticoid-induced genes is markedly enhanced by a LABA in bronchial epithelial BEAS-2B cells (Kaur et al., 2008; BinMahfouz et al., 2015; Joshi et al., 2015b; Rider et al., 2015). As similar effects occur in primary human bronchial epithelial cells (pHBECs) (Kaur et al., 2008; Moodley et al., 2013; Holden et al., 2014), a key regulator of inflammatory responses (Knight and Holgate, 2003), this mechanism may be physiologically relevant. Furthermore, there is increasing evidence that gene induction by GR (i.e., transactivation) contributes to the anti-inflammatory effects of glucocorticoids (Clark and Belvisi, 2012; Newton, 2014; Oh et al., 2017), and, in vivo, airway epithelial cells are an indispensable site of action (Klaßen et al., 2017). Indeed, analysis of inflammatory gene expression suggests that GR transactivation operates alongside transrepression to produce repression (King et al., 2012). As many genes are induced in vivo in the human airways following ICS inhalation (Kelly et al., 2012; Leigh et al., 2016), or ICS/LABA inhalation (Lee et al., 2016), transcriptional activation most likely contributes to the clinical efficacy of these drugs.

LABAs induce expression of numerous genes (Yan et al., 2018). Some, including the bronchoprotective, regulator of G protein signaling, RGS2 (Holden et al., 2011, 2014), and the anti-inflammatory genes, CD200 and CRISPLD2 (Moodley et al., 2013; Himes et al., 2014; Vaine and Soberman, 2014; BinMahfouz et al., 2015; Joshi et al., 2015b), are potentially beneficial. Others (CXCL2, IL6, IL11) may be undesirable (Ammit et al., 2002; Yan et al., 2018). Regardless, the impact of glucocorticoids on these genomic effects of LABAs is gene-dependent. For example, LABA-induced IL6 expression is repressed by glucocorticoid (Ammit et al., 2002; Holden et al., 2010), whereas RGS2, CRISPLD2, and CD200 mRNAs are synergistically enhanced (Holden et al., 2011, 2014; BinMahfouz et al., 2015; Joshi et al., 2015b). In contrast, the phosphatase, DUSP1, is induced by LABAs and glucocorticoids, and the effects of these two drugs in BEAS-2B cells and airways smooth muscle cells are additive (Kaur et al., 2008; Manetsch et al., 2012, 2013).

Patients with asthma requiring high-dose ICS, or oral corticosteroid, may suffer from multiple side effects, including oral candidiasis, hypothalamus-pituitary-adrenal axis suppression, decreased bone density, and osteoporosis. Accordingly, GR agonists showing an improved therapeutic index are urgently needed (Newton et al., 2010; Adcock et al., 2012). However, the current positioning of ICS/LABA combination therapy reveals an unmet need to elucidate the molecular

interactions between these two drugs. Such analyses should promote discovery of novel GR agonists that best capture therapeutic benefits, while avoiding undesirable outcomes. Because BEAS-2B cells behave similarly to pHBECs (Kelly et al., 2012; Moodley et al., 2013; Holden et al., 2014; Rider et al., 2015), they were used to interrogate potential mechanisms by which LABAs enhance GR-dependent transcription.

Materials and Methods

Cell Culture and Compounds. Human bronchial epithelial (BEAS-2B) cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's/Ham's F12 medium supplemented with 14 mM NaHCO₃, 2 mM L-glutamine, and 10% fetal calf serum (all Invitrogen, Burlington, ON, Canada). Cells were cultured at 37°C in 5% CO₂/95% air and were incubated in serum-free medium overnight prior to experiments. Dexamethasone and RU486 (mifepristone) (both Sigma-Aldrich, Oakville, ON, Canada), budesonide and formoterol fumarate (formoterol) (gifts from AstraZeneca Sweden, Mölndal, Sweden), salmeterol xinafoate (salmeterol) and fluticasone propionate (gifts from GlaxoSmithKline, Stevenage, UK), Org 34517 [11β-(1,3-benzodioxolo)-17β-hydroxy-17-(1-propynyl)-oestra-4,9-dien-3-one] (gift from Chiesi Farmaceutici, Parma, Italy) (Bachmann et al., 2003), and dexamethasone 21-mesylate (Dex-Mes; Steraloids, Newport, RI) were dissolved in dimethylsulfoxide (Sigma-Aldrich) Final dimethylsulfoxide concentrations on cells were ≤0.1%. Cycloheximide (Sigma-Aldrich) was dissolved in Hank's balanced salt solution (Invitrogen).

Luciferase Reporters and Assay. BEAS-2B cells stably transfected with a GRE reporter plasmid, pGL3.neo.TATA.2GRE, which contains two simple GRE sites upstream of a minimal TATA box driving luciferase expression, or pADneo2-C6-BGL, which contains six tandemly repeated cAMP response element sites upstream of a minimal β-globin promoter driving a luciferase gene, were as described (Chivers et al., 2004; Meja et al., 2004). Cells were maintained in media containing 0.25 mg/ml G-418 (Promega, Madison, WI). Cells, grown to confluence in 24-well plates, were incubated overnight in serum-free, antibiotic-free media prior to experiments. Cells were harvested in reporter lysis buffer, and luciferase assays were performed using a Firefly Luciferase Assay Kit (Biotium, Hayward, CA).

Western Blotting and Small Interfering RNA Silencing. Western blotting was performed according to standard procedures. Cells were lysed in 1× Laemmli buffer supplemented with phosphatase inhibitors (Sigma-Aldrich) and 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN), size fractionated on 12% acrylamide gels, and electrotransferred onto Hybond enhanced chemiluminescence membranes (GE Healthcare, Waukesha, WI). Membranes were blocked in 5% milk in Tris-buffered saline containing 1% Tween 20 and probed with antibodies against CREB1 (9197; Cell Signaling Technology, Danvers, MA), serine 133 phosphorylated CREB1 (9191; Cell Signaling Technology), GR (sc-8992; Santa Cruz, Dallas, TX), serine 203 (S203), serine 211 (S211) or serine 226 (S226) phosphorylated GR (ab195703, ab55189, and ab195789, respectively, from Abcam, Toronto, ON), and glyceraldehyde-3-phosphate dehydrogenase [4699-9555(ST), AbD Serotec, Raleigh, NC; or in Fig. 4B and Supplemental Fig. 6, 14C10, Cell Signaling Technology]. After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-mouse immunoglobulin (115-035-003; Jackson ImmunoResearch Laboratories). Detection was by enhanced chemiluminescence (Pierce enhanced chemiluminescence Western blotting substrate; ThermoFisher Scientific, Rockford, IL) and visualized by autoradiography. GR small interfering RNA (siRNA) 5 (target 5'-AAG TGC AAA CCT GCT GTG TTT-3') and 6 (target 5'-AAG TGC AAA CCT GCT GTG TTT-3') and control siRNA (LMNA_1, target 5'-AAC TGG ACT TCC AGA AGA

ACA-3') were purchased from Qiagen (Valencia, CA) and transfected at 25 nM into cells using RNAiMAX (Invitrogen).

Cytoplasmic and nuclear extracts were prepared by scraping cells and then resuspending cell pellets in 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, and 1× protease inhibitors on ice for 15 minutes. After centrifugation (14,000g, 5 minutes, 4°C), the supernatant-containing cytoplasm was removed and frozen at -20°C prior to Western blot analysis. The nuclear pellets were washed and resuspended in 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 1× protease inhibitors. Following repeated and vigorous agitation, nuclear debris was pelleted (14,000g, 10 minutes, 4°C), and the soluble nuclear fraction was frozen at -20°C prior to Western blot analysis.

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted (RNeasy mini kit; Qiagen), and 0.5 μg was used for cDNA synthesis (qScript kit; Quanta, Gaithersburg, MD). Following a 1:4 dilution, quantitative real-time polymerase chain reaction (qPCR) was performed on 2.5 μl cDNA in 10 μl reactions using 7900HT or StepOnePlus instruments (Applied Biosystems, Foster City, CA) and SYBR GreenER chemistry (Invitrogen). Melt curve analysis was performed to confirm primer specificity. Serial cDNA dilution was used to assess primer efficiency and to obtain relative expression levels. In later experiments, the $\Delta\Delta C_T$ method was used with validated primers. Primers for qPCR are listed in Supplemental Table 1A.

Immunofluorescent Microscopy. Cells in eight-well microscopy slides were placed on ice and fixed with 4% paraformaldehyde for 15 minutes, permeabilized (0.3% Triton-X/Hank's balance salt solution) for 10 minutes, and then blocked overnight with 10% goat serum at 4°C. Slides were incubated with primary anti-GR antibody (PA1-511A; ThermoFisher Scientific) for 1 hour, followed by 1 hour with Alexa Fluor 488 goat anti-rabbit F(ab')₂ fragment secondary antibody containing 1 μM 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted using ProLong Gold antifade reagent. Slides were visualized using an Olympus IX81 FV1000 laser-scanning confocal microscope with 40× magnification at 405 (blue) and 488 (green) nm. Exported ".oif" files were analyzed using Volocity software and Pearson product-moment correlation coefficient used to enumerate colocalization between the green (GR) and blue (DAPI) image channels.

Chromatin Immunoprecipitation. GR chromatin immunoprecipitation (ChIP) followed by massively parallel sequencing (ChIP-seq) data from control or dexamethasone (100 nM)-treated BEAS-2B cells has previously been published (Kadiyala et al., 2016). GR-binding regions were subjected to bioinformatic analysis with TFBIND (<http://tfbind.hgc.jp/>) to identify putative GRE sites (Tsunoda and Takagi, 1999). For ChIP-polymerase chain reaction (PCR) analysis, BEAS-2B cells were grown in complete medium in 100-mm cell-culture plates. Plates at 80% confluence, ~10⁷ cells, were serum starved overnight prior to treatments. Protein-DNA cross-linking was performed by adding 16% methanol-free formaldehyde (ThermoFisher Scientific) directly to the culture medium to a final concentration of 1%, and ChIP was performed in the main manuscript using an anti-GR antibody (PA1-511A; ThermoFisher Scientific) or in the Supplemental Fig. 13, as previously described (Sasse et al., 2013; Altonsy et al., 2014). Purified DNA was analyzed by qPCR performed in triplicate. Relative occupancy was calculated on a log₂ scale by the $\Delta\Delta C_T$ method. The averaged C_T for each test region was normalized to the geometric mean of the C_T value obtained for three negative control regions, as described (Sasse et al., 2013; Altonsy et al., 2014). Primer sequences used for ChIP qPCR are as listed in Supplemental Table 1B.

Microarray Analysis. BEAS-2B cells were either not treated or treated with budesonide (100 nM), formoterol (10 nM), or both combined for 1, 2, 6, and 18 hours. Total RNA quality, prepared as above, was assessed on a 2100 Bioanalyzer using RNA 6000 Nano LabChips (Agilent Technologies, Santa Clara, CA). First- and second-strand synthesis was performed with GeneChip 3' IVT Express kits (Affymetrix, Santa Clara, CA) and in vitro transcription generated biotin-labeled amplified RNA. After purification and fragmentation,

hybridization to PrimeView microarrays (Affymetrix) was for 16 hours prior to washing and scanning using a GeneChip Fluidics Station 450 and Scanner 3000 G7, respectively. Robust multiarray averaging, quantile normalization, and median polishing on logged probe set intensity values were performed using Affymetrix Expression Console software. Fold change of the probe set intensity values to the time-matched untreated controls was calculated, and one-way analysis of variance was performed using Transcriptome Analysis Console (TAC) software (Affymetrix). Data files are deposited with National Center for Biotechnology Information's Gene Expression Omnibus [accession GSE115830 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115830>)]. Where genes are represented by multiple probe sets, only those with the greatest overall change were retained for subsequent analyses.

Statistical Analyses and Curve Fitting. Data from *N* separate determinations are plotted as means ± S.E. Statistical analyses generally assumed data to be normally distributed (Prism version 6.01; GraphPad Software, San Diego, CA). In some experiments, different batches of cells gave rise to different levels of reporter, or gene, induction. In such cases, nonparametric statistics were necessarily used. The tests used are stated in the figure legends. Four-parameter curve fitting of agonist concentration-response data was performed in Prism version 6.01 to produce values for the maximum achievable response (E_{Max}) and pEC₅₀, which is defined as the negative log of the molar EC₅₀. Schild analysis to produce pA₂ values, defined as the negative log of the molar concentration of antagonist that is required to double the concentration of agonist to achieve the original response, was performed in Prism 6.01. Operational modeling (Leff et al., 1990) was performed in Prism 6.01 to produce pK_A, negative log of the molar affinity, values using the method of fractional irreversible receptor depletion, as previously described (Joshi et al., 2015b).

Results

Effect of Glucocorticoid on Phospho-CREB and cAMP Response Element-Dependent Transcription Induced by LABA. β₂-Adrenoceptor agonists enhance GR-dependent transcription via a β₂-adrenoceptor-mediated mechanism that is sensitized by PDE4 inhibition and involves the classic cAMP-protein kinase A (PKA) pathway (Kaur et al., 2008; Moodley et al., 2013; BinMahfouz et al., 2015). To assess effects of glucocorticoid on this pathway, BEAS-2B cells were treated with a maximally effective concentration (10 nM) of the LABA, formoterol, in the absence and presence of a maximally effective concentration, 1 μM, of dexamethasone (Kaur et al., 2008; Rider et al., 2011). Formoterol induced phosphorylation of the cAMP-activated transcription factors, CREB1 and ATF1, within 10 minutes (Fig. 1A). This was maintained at 30 minutes, before time-dependently declining. There were no effects of dexamethasone cotreatment, and expression of total CREB1 was unaltered over this time frame (Fig. 1A; Supplemental Fig. 1). BEAS-2B cells harboring a 6×CRE luciferase reporter were stimulated with various concentrations of formoterol in the absence or presence of 1 μM dexamethasone (Fig. 1B). Formoterol maximally induced 6×CRE-dependent luciferase activity by 19.6 ± 0.6-fold with a pEC₅₀ of 10.3 ± 0.1. Dexamethasone modestly reduced the E_{Max} for formoterol to 18.0 ± 0.6-fold, whereas the pEC₅₀ remained unchanged at 10.3 ± 0.1 (Fig. 1B). Thus, glucocorticoid had little effect on the activation of cAMP-dependent transcription by β₂-adrenoceptor agonist.

Enhancement of Glucocorticoid-Induced Simple GRE Transcription by LABA Requires GR and Is Time-Dependent. In BEAS-2B cells harboring a simple

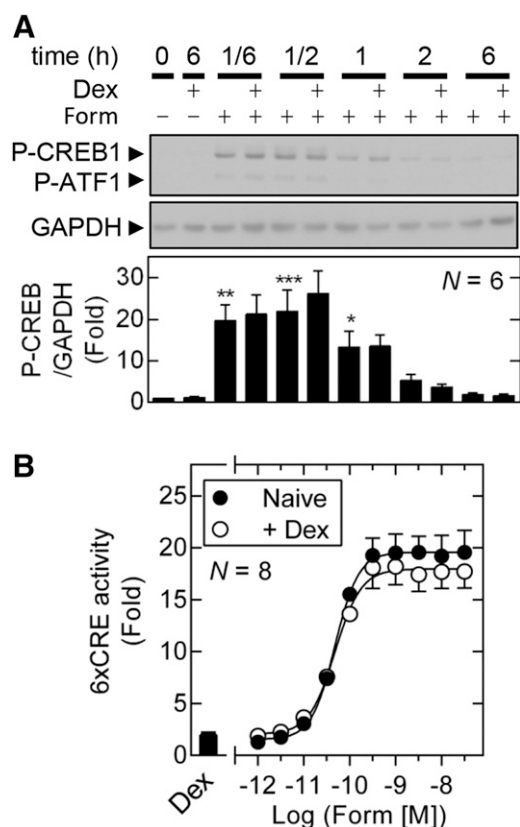


Fig. 1. Effect of glucocorticoid on transcriptional activation by LABA. (A) BEAS-2B cells were treated with formoterol (Form; 10 nM) and/or dexamethasone (Dex; 1 μ M) for the times indicated prior to harvesting for Western blot analysis of serine 133-phosphorylated CREB1 (P-CREB1) and glyceraldehyde-3-phosphate dehydrogenase. Phosphorylated ATF1 (P-ATF1), as indicated, was also detected. Data for total CREB appear as Supplemental Fig. 1. Blots representative of N independent experiments are shown. Following densitometric analysis, data for P-CREB1 were normalized to glyceraldehyde-3-phosphate dehydrogenase, expressed as fold of untreated, and are plotted as mean \pm S.E. Statistical significance comparing each formoterol treatment time to $t = 0$ and to the effect of Dex + Form, at each time, was tested by analysis of variance with Dunn's multiple comparisons test. Compared with $t = 0$: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) BEAS-2B cells harboring a 6 \times CRE luciferase reporter were treated with various concentrations of formoterol in the absence or presence of dexamethasone (1 μ M). Cells were harvested after 6 hours for luciferase assay. Data from N independent experiments are expressed as fold of untreated and are plotted as mean \pm S.E.

2 \times GRE luciferase reporter, dexamethasone increased luciferase activity in a concentration-dependent manner (pEC_{50} 7.7 ± 0.2 ; E_{Max} 10.6 ± 1.1 -fold) (Fig. 2A). Alone, formoterol (10 nM) had no effect on 2 \times GRE-dependent luciferase activity, but enhanced the maximal response to dexamethasone to 31.9 ± 3.6 -fold without changing the potency (pEC_{50} 7.7 ± 0.2) (Fig. 2A). Reporter activity induced by dexamethasone and dexamethasone plus formoterol was competitively inhibited by the GR antagonist, Org34517 (Supplemental Fig. 2), with pA_2 values of 8.4 ± 0.1 and 8.5 ± 0.1 , respectively. This is consistent with GR-mediated responses (King et al., 2012; Joshi et al., 2015b). A single concentration of Org34517 (1 μ M) produced near-complete inhibition of the responses to maximally effective concentrations of dexamethasone (Fig. 2B) and the ICSs, budesonide and fluticasone propionate (Supplemental Fig. 2D). The LABAs, formoterol and salmeterol, enhanced glucocorticoid-induced 2 \times GRE reporter activity, and this was

also prevented by Org34517 (Fig. 2B; Supplemental Fig. 2D). To confirm GR dependence, two independent siRNAs, which produced a near-complete loss of GR, abolished 2 \times GRE reporter activation induced by budesonide and budesonide plus formoterol (Supplemental Fig. 3).

The effect of time of LABA addition on the enhancement of GRE-dependent transcription was investigated. BEAS-2B 2 \times GRE reporter cells were treated with a maximally effective concentration of dexamethasone (1 μ M) and formoterol (10 nM) added at various times prior to, with, or after the glucocorticoid (Fig. 2C). Reporter activity induced by dexamethasone was maximally enhanced by formoterol cotreatment (i.e., addition time of 0), or when formoterol was added 15 minutes prior to, or after, the dexamethasone addition. Increasing the formoterol addition time either prior to, or after, dexamethasone addition reduced enhancement of the 2 \times GRE reporter by formoterol (Fig. 2C). Therefore, cotreatment is necessary for maximal cooperativity and is consistent with a rapidly acting pathway to enhance simple GR-mediated GRE-dependent transcription.

LABA Enhancement of GRE-Dependent Transcription Occurs Early and Is Insensitive to Protein Synthesis Inhibition. Following dexamethasone treatment of BEAS-2B cells harboring the 2 \times GRE reporter, luciferase activity time-dependently accumulated to a maximum at 4–6 hours, after which luciferase activity declined modestly (Fig. 2D). Such data are replicated with other GR ligands and collectively suggest an early pulse of GRE-dependent transcription (BinMahfouz et al., 2015; Joshi et al., 2015a), which is largely abrogated by 4–6 hours. This was confirmed in BEAS-2B cells harboring the 2 \times GRE reporter, where luciferase mRNA revealed a rapid glucocorticoid-induced kinetic with peak expression at 2 hours, before returning to near baseline at 5–6 hours (Supplemental Fig. 4A). Thus, peak luciferase activity accumulation, 4–6 hours postglucocorticoid, is primarily due to enhanced mRNA expression during the first 4 hours post-glucocorticoid administration. Formoterol enhanced this initial rate of dexamethasone-induced luciferase production, as revealed by the initial (0–4 hours) slope of luciferase activity accumulation (Fig. 2D). Indeed, examination of the percentage enhancement produced by formoterol revealed a clear effect 1 hour post-treatment (Fig. 2D, lower panel). Thus, not only did glucocorticoid-induced 2 \times GRE-dependent transcription occur over a relatively short window of time, but LABA enhancement was confined to the same early time frame. Indeed, at 2 hours, when formoterol alone showed little effect on luciferase mRNA accumulation, dexamethasone-induced luciferase mRNA expression was enhanced 2- to 3-fold by formoterol (Fig. 2E, upper panel, left axis).

To examine the effect of protein synthesis inhibition on the enhancement of 2 \times GRE activity by formoterol, cycloheximide was first tested on the induction of luciferase mRNA by dexamethasone (Supplemental Fig. 4B). Alone, cycloheximide had no effect on luciferase mRNA expression, but enhanced expression induced by dexamethasone at 1 and 2 hours. This persisted until 6 hours, despite dexamethasone-induced luciferase mRNA having returned to basal levels (Supplemental Fig. 4B). Although not investigated further, these data raise the prospect that either simple GRE-dependent transcription is negatively regulated by a labile inhibitor that requires ongoing resynthesis or dexamethasone rapidly induces the expression of a negative regulator of GRE-dependent

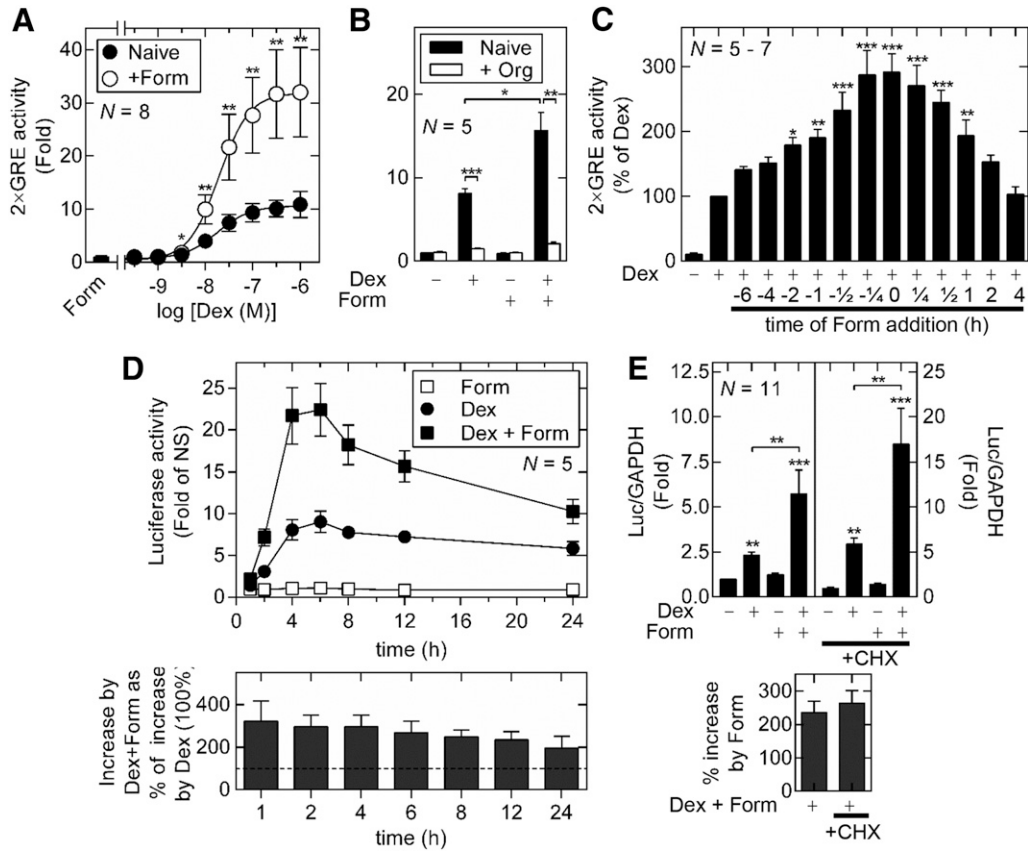


Fig. 2. Formoterol rapidly enhances GR-mediated simple GRE-dependent transcription. (A) BEAS-2B cells harboring a 2×GRE luciferase reporter (BEAS-2B 2×GRE cells) were treated with formoterol (Form; 10 nM) and/or the indicated concentrations of dexamethasone (Dex). After 6 hours, cells were harvested for luciferase assay. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± S.E. Statistical significance for the fold induction between naive and formoterol-treated was evaluated by Wilcoxon matched-pairs signed rank test at each dexamethasone concentration. **P* < 0.05; ***P* < 0.01. (B) BEAS-2B 2×GRE cells were pretreated with Org34517 (Org; 1 μM), or not, prior to stimulation with the dexamethasone (Dex; 1 μM) in the absence or presence of formoterol (Form; 10 nM). After 6 hours, cells were harvested for luciferase assay. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± S.E. Statistical significance for the fold induction between the indicated treatments was tested by analysis of variance with Bonferroni's correction. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. (C) BEAS-2B 2×GRE cells were treated with dexamethasone (1 μM). Formoterol (10 nM) was added either with the dexamethasone (*t* = 0) or at various times prior (−6, −4, −2, −1, −1/2, or −1/4 hours) to or at various times after (1/4, 1/2, 1, 2, or 4 hours) the addition of dexamethasone. In each case, cells were harvested for luciferase assay 6 hours after the addition of dexamethasone. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± S.E. Statistical significance relative to dexamethasone was examined by analysis of variance with a Dunnett's post-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. (D) BEAS-2B 2×GRE reporter cells were treated with dexamethasone (1 μM), formoterol (10 nM), or the combination (Dex + Form). Cells were harvested at the indicated times for luciferase assay. Data (upper panel), from *N* independent experiments, are plotted as fold of no stimulation (NS) as means ± S.E. To more clearly show the enhancement by formoterol at each time (lower panel), the absolute increase (over NS) produced by dexamethasone + formoterol was expressed as a percentage of the absolute increase (over NS) produced by dexamethasone alone (100%, dotted line) and is plotted as mean ± S.E. (E) BEAS-2B 2×GRE reporter cells were treated with dexamethasone (1 μM) in the absence or presence of formoterol (10 nM) and/or cycloheximide (CHX; 100 μg/ml), as indicated. Cells were harvested after 2 hours for qPCR analysis of luciferase (Luc) and glyceraldehyde-3-phosphate dehydrogenase mRNA. Data (upper panel) from *N* independent experiments, expressed as Luc/glyceraldehyde-3-phosphate dehydrogenase, are plotted as fold of untreated as means ± S.E. Statistical significance relative to untreated control, or cycloheximide alone, as appropriate, was examined by analysis of variance with a Dunn's post-test. To evaluate the effect of formoterol on the induction by dexamethasone, each in the absence or presence of cycloheximide, fold data were analyzed by Wilcoxon matched-pairs signed rank test. ***P* < 0.01; ****P* < 0.001. Data were also replotted (lower panel) to show the effect of formoterol on the induction of luciferase RNA by dexamethasone in the absence or presence of CHX. In each case, the data are expressed as a percentage of dexamethasone or dexamethasone + cycloheximide, as appropriate. Comparing without and with cycloheximide, no significant difference in this percentage was apparent as assessed by paired *t* test.

transcription. The effect of cycloheximide on the enhancement of dexamethasone-induced transcription by formoterol was tested. At 2 hours dexamethasone-induced luciferase mRNA expression was enhanced 2- to 3-fold by formoterol, which alone was without effect (Fig. 2E, upper panel, left axis). In the presence of cycloheximide, dexamethasone produced an elevated level of luciferase mRNA that was also enhanced 2- to 3-fold by formoterol (Fig. 2E, upper panel, right axis). Thus, formoterol enhanced glucocorticoid-induced 2×GRE-dependent transcription to similar extents, whether in the absence or presence of cycloheximide (Fig. 2E, lower panel).

These data support a directly acting pathway that does not involve LABA-induced de novo protein synthesis to enhance GR-mediated transcription.

LABAs Do Not Affect GR Expression or GR Phosphorylation. BEAS-2B cells were treated with dexamethasone in the absence or presence of LABAs prior to analysis of GR expression. As shown in Fig. 3A, treatment with glucocorticoid decreased GR protein expression. This effect was apparent at 6 hours and was more pronounced by 18 hours (Fig. 3A). However, maximally effective concentrations of formoterol and salmeterol, either alone or in combination

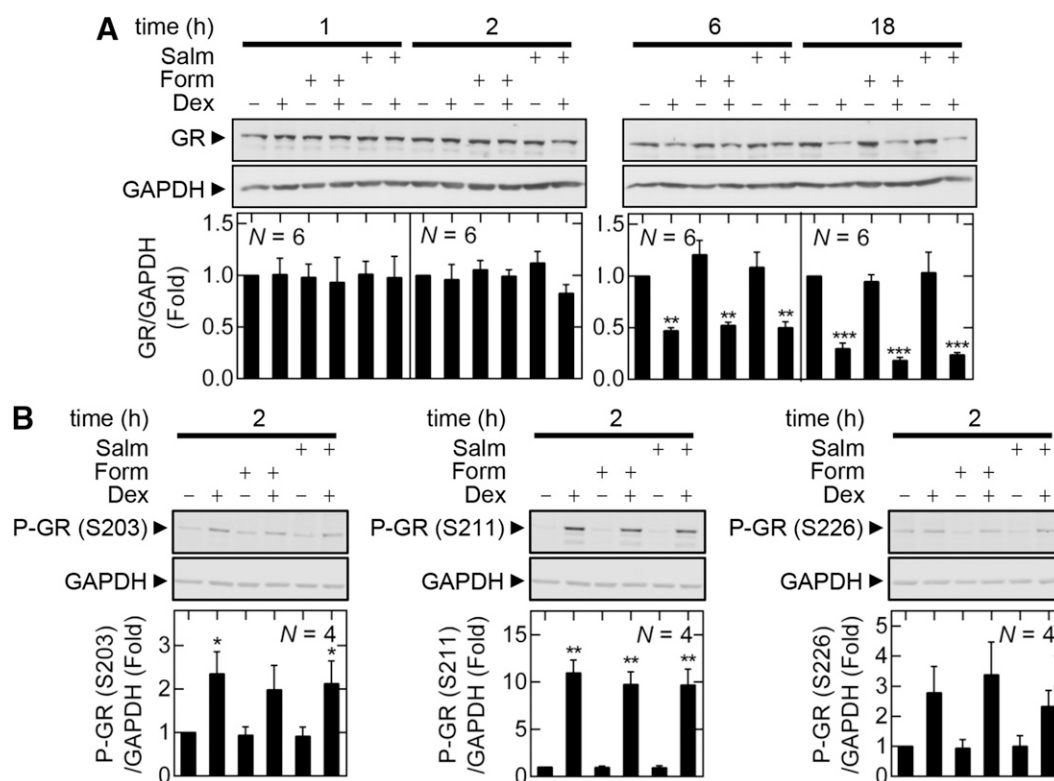


Fig. 3. No effect of LABAs on the glucocorticoid-dependent loss of GR expression or on GR phosphorylation. (A) BEAS-2B cells were either not treated or treated with dexamethasone (Dex; 1 μ M), formoterol (Form; 10 nM), or salmeterol (Salm; 100 nM) alone or in combination. Cells were harvested at the indicated times prior to Western blot analysis for GR and glyceraldehyde-3-phosphate dehydrogenase. Blots representative of *N* independent experiments are shown. Following densitometric analysis, data, normalized to glyceraldehyde-3-phosphate dehydrogenase, were expressed as fold of untreated at each time and are plotted as mean \pm S.E. Statistical significance compared with untreated control was tested by analysis of variance with a Dunnett's post-test. $**P \leq 0.01$; $***P \leq 0.001$. (B) BEAS-2B cells were treated with dexamethasone (1 μ M) and/or the LABAs formoterol (10 nM) or salmeterol (100 nM). After 2 hours, the cells were harvested for Western blot analysis of S203, S211, and S226 phosphorylated GR (P-GR) and glyceraldehyde-3-phosphate dehydrogenase. Densitometric data from *N* independent experiments were normalized to glyceraldehyde-3-phosphate dehydrogenase, expressed as fold of untreated, and are plotted as means \pm S.E. Statistical significance compared with untreated and between dexamethasone and dexamethasone plus LABAs was tested by analysis of variance with Bonferroni's correction. Compared with untreated: $*P \leq 0.05$; $**P \leq 0.01$.

with dexamethasone, had no effect on GR protein expression (Fig. 3A). These data were confirmed at 6 hours using budesonide/formoterol and fluticasone propionate/salmeterol combinations (Supplemental Fig. 5A). Similarly, GR mRNA expression was significantly reduced following 6-hour treatments of BEAS-2B cells with glucocorticoids, and this was unaffected by formoterol cotreatment (Supplemental Fig. 5B). Alone, formoterol modestly, 1.21 ± 0.04 -fold, increased GR mRNA.

Phosphorylation of GR at serines 203, 211, and 226 (Ser²⁰³, Ser²¹¹, and Ser²²⁶) has been previously characterized and, in the case of Ser²¹¹, is maximal 1–2 hours following exposure to glucocorticoid (Avenant et al., 2010). In initial analyses, 2 hours of dexamethasone (1 μ M) induced phosphorylation at each site, with increases at Ser²¹¹ being most robust (Fig. 3B). Similar data were obtained at 1 hour (Supplemental Fig. 6B). Following siRNA silencing to reduce GR expression, the signal detected by each phospho-specific antibody was markedly reduced (Supplemental Fig. 6A). This confirms specificity for GR. Although the dexamethasone-induced phosphorylation was not significant for Ser²²⁶, combining these results (*N* = 4) with the data (*N* = 4) from Supplemental Fig. 6C, for the dexamethasone, formoterol, dexamethasone plus formoterol treatments, confirmed that glucocorticoid-enhanced phosphorylation at Ser²²⁶ was also significant (*N* = 8,

Bonferroni's multiple comparison test $P \leq 0.05$). In each case, there was no effect of maximally effective concentrations of either formoterol or salmeterol on phosphorylation at these three sites at either 1 or 2 hours (Fig. 3B; Supplemental Fig. 6B). Although dexamethasone concentration-dependently increased GR phosphorylation at each site, no effects of formoterol were apparent (Supplemental Fig. 6C).

LABAs Do Not Affect Glucocorticoid Affinity (K_A) for GR. The effect of formoterol (10 nM) on the affinity of budesonide for GR in 2 \times GRE BEAS-2B reporter cells was determined using the method of fractional, irreversible receptor depletion, as previously described for GR (Joshi et al., 2015b). Agonist concentration–response curves were constructed in cells that had been treated for 30 minutes, or not, with the alkylating agent Dex-Mes (10 nM) (Simons and Thompson, 1981), prior to operational model fitting (Leff et al., 1990) (Supplemental Fig. 7). Budesonide produced a maximal response (E_{Max}), as defined by the upper asymptote of the concentration–response curve, of 6.69-fold (Table 1). This was increased to 13.90-fold in the presence of formoterol, without material change, 8.50 and 8.40, respectively, to the pEC_{50} (Table 1). Pretreatment with Dex-Mes depressed the E_{Max} produced by budesonide and budesonide plus formoterol by 37.5% and 41.7%, respectively, without

TABLE 1

Operational modeling using 2×GRE-dependent transcription to calculate budesonide affinity (K_A) in absence and presence of LABA

BEAS-2B 2×GRE reporter cells were either not treated or treated with 10 nM Dex-MES for 30 minutes. The medium was then removed, and the cells were rinsed with serum-free medium prior to treatment with various concentrations of budesonide, each in the absence or presence of formoterol (10 nM). After 6 hours, the cells were harvested for luciferase assay. Data are plotted in Supplemental Fig. 7. GraphPad Prism 6.01 was used to generate parameters from concentration–response curves. Operational model fitting (Leff et al., 1990), as applied to GR (Joshi et al., 2015b), was used to provide pK_A values for budesonide in the absence and presence of formoterol. The K_A/EC_{50} ratio being less than 1 indicates that there is no receptor reserve.

	Treatments			
	Budesonide		Budesonide + Formoterol	
	Naive	+ Dex-MES	Naive	+ Dex-MES
E_{Max} (fold)	6.69 ± 0.27	4.18 ± 0.26	13.90 ± 0.65	8.11 ± 0.37
pEC_{50}	8.50 ± 0.09	8.34 ± 0.13	8.40 ± 0.11	8.26 ± 0.11
pK_A		8.99 ± 0.82		8.73 ± 0.62
K_A/EC_{50}	0.32	—	0.47	—

affecting potency (Table 1). Operational modeling of these response curves produced functionally derived pK_A values of 8.99 and 8.73 for budesonide in the absence and presence of formoterol, respectively (Table 1). Thus, formoterol did not alter the affinity of budesonide for GR.

LABAs Do Not Affect Glucocorticoid-Induced Nuclear Translocation of GR. Although enhanced GR nuclear translocation has been proposed to explain how LABAs enhance the therapeutic activity of ICS (Eickelberg et al., 1999; Roth et al., 2002; Usmani et al., 2005; Mortaz et al., 2008; Haque et al., 2013), other studies do not support this (Gruol and Altschmied, 1993; Lovén et al., 2007). To address this possibility, BEAS-2B cells were treated with a maximally effective concentration of dexamethasone prior to immunofluorescent imaging of GR. In untreated cells, GR was distributed diffusely throughout the cytosol, whereas, following dexamethasone addition, translocation to the nucleus was evident by 15 minutes and was maintained for at least 6 hours (Supplemental Fig. 8A). Salmeterol (100 nM) did not promote translocation of GR to the nucleus at 15 or 30 minutes, or 1, 2, 4, or 6 hours (Supplemental Fig. 8B). Further analysis at 1 hour confirmed these observations; using Pearson's correlation to assess the overlap between fluorescein isothiocyanate-labeled GR- and DAPI-stained nuclei revealed no difference in the localization of GR between cells treated with dexamethasone or dexamethasone plus salmeterol (Fig. 4A).

Differential lysis was used to separate nuclear and cytoplasmic fractions of BEAS-2B cells. The effectiveness of separation was confirmed by Western blot analysis of CREB1, a constitutively expressed nuclear transcription factor, and glyceraldehyde-3-phosphate dehydrogenase, a cytoplasmic enzyme (Fig. 4B). Western blotting showed GR in the cytoplasmic extracts of untreated cells, but 1 hour after fluticasone propionate or budesonide treatment there was a loss of cytoplasmic GR, with a corresponding increase of GR in the nuclear fractions (Fig. 4B). In contrast, salmeterol and formoterol alone were inactive and had no effect on the glucocorticoid-induced redistribution of GR (Fig. 4B). Similar data were produced at 2 hours (Fig. 4B).

Mifepristone (RU486), which promotes nuclear translocation of GR (Chivers et al., 2004; Lewis-Tuffin et al., 2007), was essentially inactive (E_{Max} 1.6 ± 0.3-fold) on the 2×GRE

reporter in BEAS-2B cells (Supplemental Fig. 9A). In the presence of formoterol (10 nM), RU486 concentration-dependently increased 2×GRE reporter activity (E_{Max} 3.52 ± 1.45-fold), and this effect was mimicked by salmeterol (100 nM), salbutamol (1 μM), and the adenylyl cyclase activator, forskolin (10 μM) (Supplemental Fig. 9B). Although RU486 produced GR nuclear translocation, there was no additional effect of cotreatment with either salmeterol or formoterol, as determined by immunofluorescence microscopy or by cell fractionation combined with Western blotting (Supplemental Fig. 9, C and D). Thus, gross changes in GR localization cannot account for the ability of β₂-adrenoceptor agonists to convert RU486 into a weak partial agonist.

GR Recruitment Does Not Explain Glucocorticoid/LABA Synergy at the 2×GRE Reporter. BEAS-2B 2×GRE cells were treated with a maximally effective concentration of budesonide in the absence and presence of formoterol prior to GR chromatin immunoprecipitation (ChIP). Initial analysis focused on a previously validated GR binding site located within an intronic region of FKBP5 (Fig. 5A) (Kadiyala et al., 2016), a gene that is highly glucocorticoid-induced, but not modulated by LABA (Kaur et al., 2008). ChIP qPCR primers were also designed to flank the two GRE sites in pGL3.2×GRE-luc.neo, the plasmid used to generate the stably transfected BEAS-2B 2×GRE cells (Fig. 5B) (Chivers et al., 2004; Kaur et al., 2008). Representative qPCR traces for FKBP5 and 2×GRE-luc are shown (Fig. 5C). There was no effect of formoterol alone on GR enrichment at these regions, but budesonide significantly enriched GR at the FKBP5 intronic region and the 2×GRE-luc construct (Fig. 5, C and D). In the context of budesonide, formoterol was without further effect. Although these data are consistent with FKBP5 mRNA not being affected by LABA (Kaur et al., 2008) (plus see Fig. 6), they indicate that increased GR recruitment to the simple 2×GRE in pGL3.2×GRE-luc does not account for the formoterol-induced enhancement of GRE-dependent transcription.

Effect of LABA on Glucocorticoid-Induced Gene Expression. As is described in a related manuscript (in preparation), BEAS-2B cells were treated with budesonide (100 nM) and formoterol (10 nM) alone or in combination for 1, 2, 6, and 18 hours prior to expression analysis using Affymetrix PrimeView arrays. Following whole array normalization, data from four independent experiments were combined, and probe sets returning ≥2-fold or ≤0.5-fold change, with $P ≤ 0.05$, for any treatment were used for subsequent analyses. Whereas both formoterol and budesonide each increased and decreased gene expression, the current manuscript focuses on genes induced (≥2-fold, $P ≤ 0.05$) by budesonide. These mRNAs showed a large range of inducibility by budesonide at each time, with the effects of formoterol varying from marked enhancement through to repression, with many mRNAs simply being unaffected. To illustrate these differences, heat maps were generated for budesonide-induced genes (budesonide: ≥2-fold, $P ≤ 0.05$; but <2-fold formoterol) that were also induced ≥4-fold by budesonide or budesonide plus formoterol (Supplemental Fig. 10). Budesonide or budesonide plus formoterol induced peak mRNA expression in a gene- and time-dependent manner. In combination (compared with budesonide treated as 100%), formoterol maximally enhanced the expression of budesonide-induced genes to 724%, 406%, 385%, and 352%, at 1, 2, 6, and 18 hours,

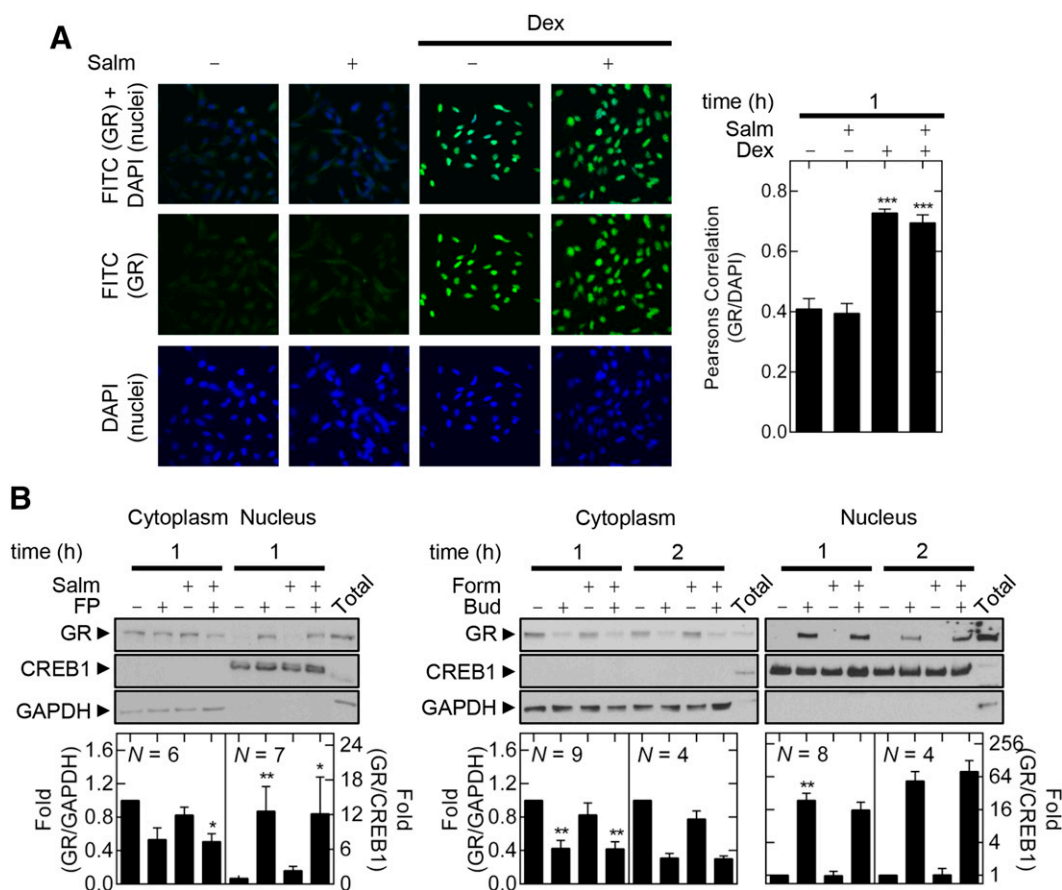


Fig. 4. No effect of LABAs on nuclear translocation by GR. (A) BEAS-2B cells at ~70% confluence were either not stimulated or treated for 1 hour with dexamethasone (Dex; 1 μ M), salmeterol (Salm; 100 nM), or both drugs in combination. Slides were fixed and probed for GR (green), and the nuclei counterstained with DAPI (blue), as described for Supplemental Fig. 8. (Right panel) Correlation between Alexa 488 (GR) staining and DAPI (nuclei) was performed using Perkin Elmer Velocity software (Waltham, MA). Analysis was performed on all cells in five images for each treatment. Data, expressed as the Pearson correlation, are presented as mean \pm S.E. Statistical significance was tested between untreated and dexamethasone, formoterol, and dexamethasone + formoterol and between dexamethasone and dexamethasone + formoterol using ANOVA with Bonferroni's correction. *** $P < 0.001$ versus untreated. (B) BEAS-2B cells were treated with fluticasone propionate (FP; 100 nM) and/or salmeterol (100 nM) or budesonide (Bud; 100 nM) and/or formoterol (Form; 10 nM) for 1 or 2 hours, as indicated. Cytoplasmic and nuclear extracts were prepared and subjected to Western blotting for GR, CREB1 (nuclear), and glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic). A total cell lysate was used as a positive control (Total). Blots representative of N such experiments are shown. Densitometry was performed on the cytoplasmic (GR/glyceraldehyde-3-phosphate dehydrogenase) and nuclear (GR/CREB1) extracts to generate the figures shown in lower panels. Data, expressed as fold, are plotted as means \pm S.E. Multiple comparison testing between each treatment and untreated and between treatments with glucocorticoid and glucocorticoid plus LABA was performed by analysis of variance with a Dunn's post-test. Compared with untreated: * $P \leq 0.05$; ** $P \leq 0.01$.

respectively (Supplemental Fig. 10). The different mRNA kinetics are depicted for selected genes showing either enhancement by formoterol (1- or 2-hour peak: PTGS2, TNFAIP3; PLAT; 6-hour peak: ZFAND, PDK4, CDKN1C, KLF15; 18-hour peak: ADH1B, CNR1), no/little effect of formoterol (1- or 2-hour peak: KLF6, CEBPB, NFKBIA; 6-hour peak: TSC22D3, FKBP5; 18-hour peak: MAOA), or repression by formoterol (6-hour peak: SLC16A12; 18-hour peak: SAA1) (Fig. 6A). In each case, qPCR (Supplemental Fig. 11A), or prior publications using glucocorticoid plus LABA combinations (Kaur et al., 2008; BinMahfouz et al., 2015; Joshi et al., 2015b; Altonsy et al., 2017), confirmed these data.

Examination of the heat maps in Supplemental Fig. 10 shows that many of the budesonide-induced mRNAs, which are enhanced by formoterol cotreatment, were also modestly induced by formoterol alone (albeit falling below the predetermined 2-fold threshold). Similarly, the microarray data reveal many mRNAs to be induced by both budesonide and formoterol (manuscript in preparation). Those mRNAs

with ≥ 2 -fold ($P \leq 0.05$) for each of budesonide and formoterol alone, but which also meet the additional criteria of being ≥ 4 -fold for any treatment, were therefore assembled into heat maps according to the time of peak expression and ranked according to the effect of the budesonide plus formoterol treatment (Supplemental Fig. 12). Various interactions between the LABA and glucocorticoid are apparent. Multiple mRNAs showed predominantly budesonide-induced expression that was enhanced by formoterol (1- or 2-hour peak: KLF4, SLC19A2; 6-hour peak: SLC16A14), whereas others were predominantly formoterol-induced and enhanced by budesonide (2-hour peak: CD200) (Fig. 6B). In addition, there were predominantly LABA-induced (2-hour peak: RGS2; 6-hour peak: CRISPLD2) or glucocorticoid-induced (6-hour peak: SLC16A14) mRNAs, in which each stimulus alone produced a relatively modest response relative to the combination, but which yielded a marked synergy that occurred either early or later in the expression kinetic (Fig. 6B). However, glucocorticoid plus separate LABA inducibility does not necessarily produce

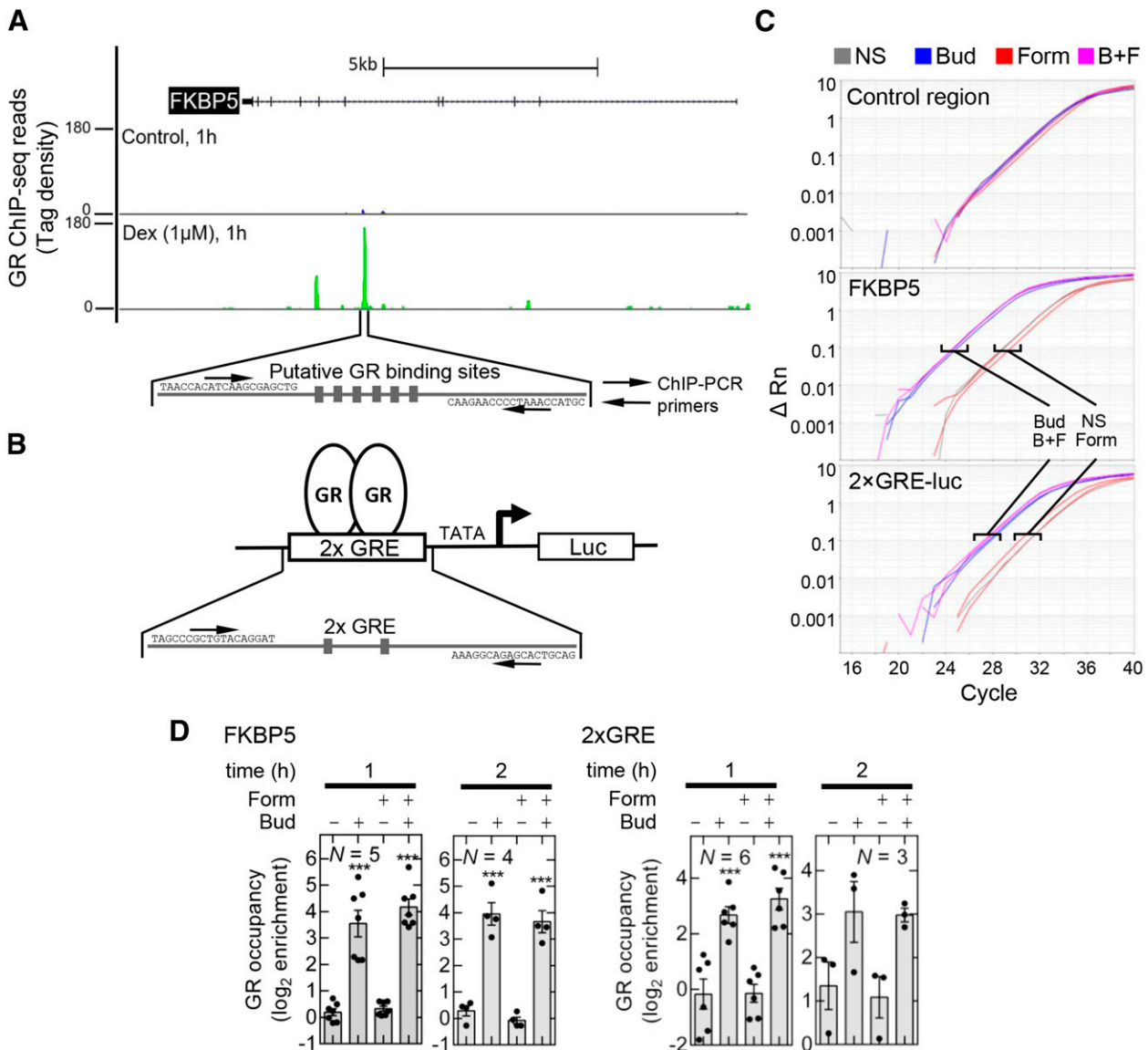


Fig. 5. Recruitment of GR to GRE is not affected by LABA. (A) BEAS-2B cells were treated for 1 hour with dexamethasone (Dex; 0.1 μ M) or vehicle control. ChIP-seq analysis for GR revealed robust GR DNA sites within intronic regions of the glucocorticoid-induced gene, FKBP5 (Kadiyala et al., 2016). Multiple putative GREs were identified by TFBIND, and qPCR primers were designed to flank these sites. (B) Schematic showing the 2 \times GREs cloned upstream of a TATA box and transcription start driving the luciferase gene in the pGL3.2 \times GRE-luc.neo plasmid used to generate the BEAS-2B 2 \times GRE-luc reporter cells. As shown, qPCR primers were designed to flank the 2 \times GRE. (C) BEAS-2B cells were either not stimulated (NS) or treated with formoterol (Form; 10 nM), budesonide (Bud; 100 nM), or both combined (B + F). ChIP was performed using an anti-GR antibody, and qPCR was performed for three unrelated control regions of genomic DNA, FKBP5, and the 2 \times GRE of pGL3.2 \times GRE-luc.neo. Representative qPCR traces are shown for genomic DNA near the MYOD gene (control region), FKBP5 and 2 \times GRE-luc. (D) Data from N experiments are shown for FKBP5 and 2 \times GRE-luc normalized to the control regions and are plotted as log₂ fold. Statistical significance was tested between untreated and budesonide, formoterol, and budesonide + formoterol and between budesonide and budesonide + formoterol using analysis of variance with Bonferroni's correction. *** P < 0.001 versus untreated. There were no significant differences between budesonide and budesonide + formoterol.

synergistic interactions. Many mRNAs revealed simple additivity (DUSP1, CEBPD), and others (PER1, KLF9) showed glucocorticoid inducibility that was essentially unchanged by LABA, this despite being LABA induced. Each of these expression profiles is validated either in the current study (Supplemental Fig. 11B) and/or in prior reports (Kaur et al., 2008; Holden et al., 2014; BinMahfouz et al., 2015; Joshi et al., 2015b).

Effect of LABA on GR Recruitment to Glucocorticoid-Induced Genes. To explore the effect of LABA on GR recruitment, ChIP-seq data were used to identify

dexamethasone-induced GR binding sites at, or near to, multiple loci for glucocorticoid-regulated genes in BEAS-2B cells (Kadiyala et al., 2016). Many mRNAs (PTGS2, KLF4), which revealed robust glucocorticoid plus LABA synergy, had no major GR binding sites (GBSs) closely associated (≤ 100 kb) with their gene loci (Supplemental Fig. 13A). Other genes (*PLAT* or *RGS2*) revealed only weak GBSs and/or were located in gene-dense regions (data not shown). In such instances, direct regulation by GR cannot be assumed. However, multiple other glucocorticoid-induced gene loci revealed robust GBSs that were variously intronic (*CRISPLD2*, *FKBP5*, *KLF15*, *NFKBIA*, *TNFAIP3*, *SLC16A12*), closely

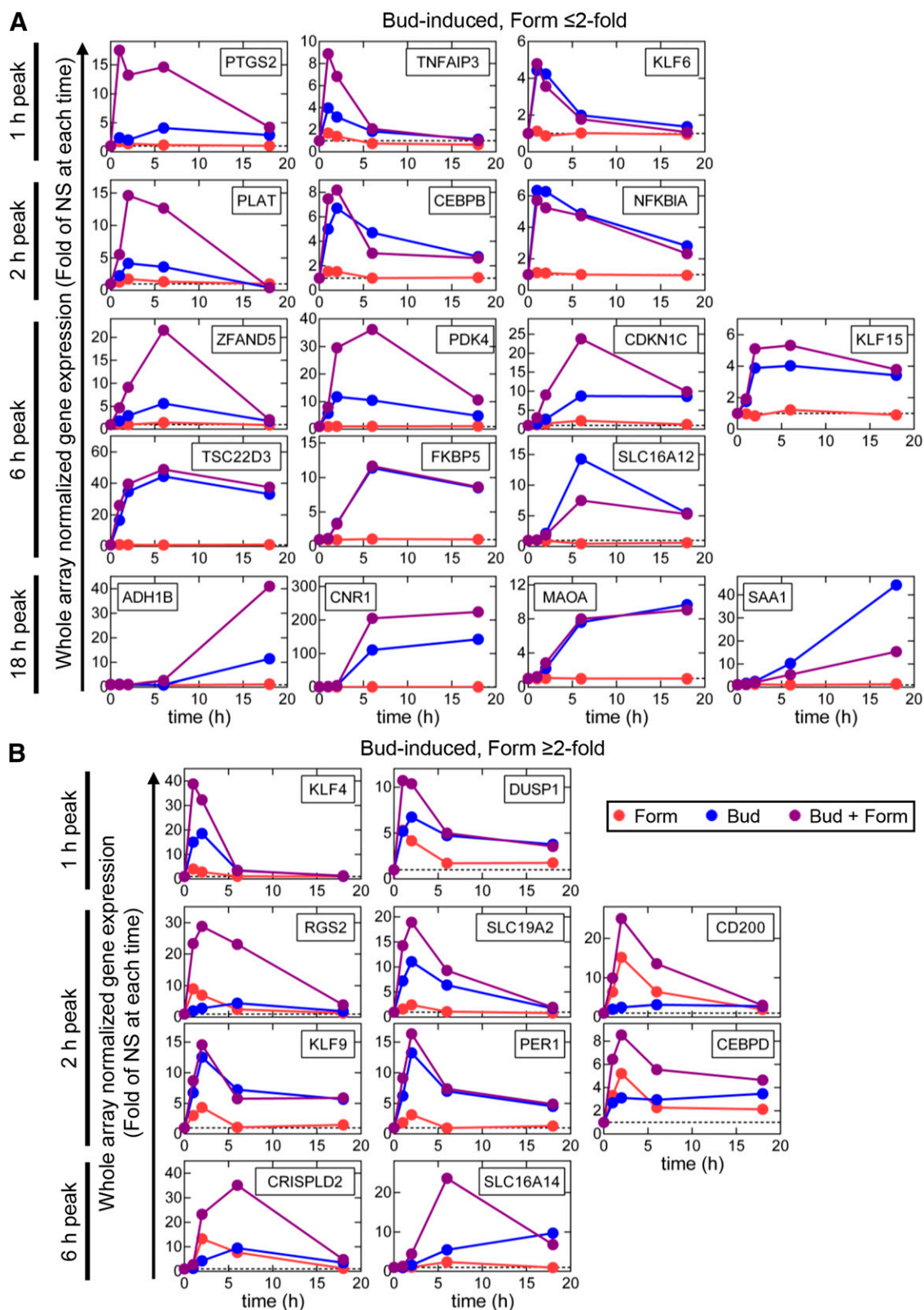


Fig. 6. Effect of formoterol on budesonide-induced gene expression. BEAS-2B cells were either not stimulated (NS) or treated with budesonide (Bud; 100 nM), formoterol (Form; 10 nM), or both together (Bud + Form). At 1, 2, 6, and 18 hours, RNA was extracted and microarray analysis was performed. (A) mRNAs that were induced ≥ 2 -fold ($P \leq 0.05$) by budesonide, but not by formoterol alone (< 2 -fold), were selected from Supplemental Fig. 10 and are presented according to the time (1, 2, 6, or 18 hours) of peak mRNA expression. (B) mRNAs that were induced ≥ 2 -fold ($P \leq 0.05$) by budesonide and by formoterol were selected from Supplemental Fig. 12 and are presented according to the time (1, 2, 6, or 18 hours) of peak mRNA expression. In both (A and B), combined array data ($N = 4$) are plotted as fold of NS for each time.

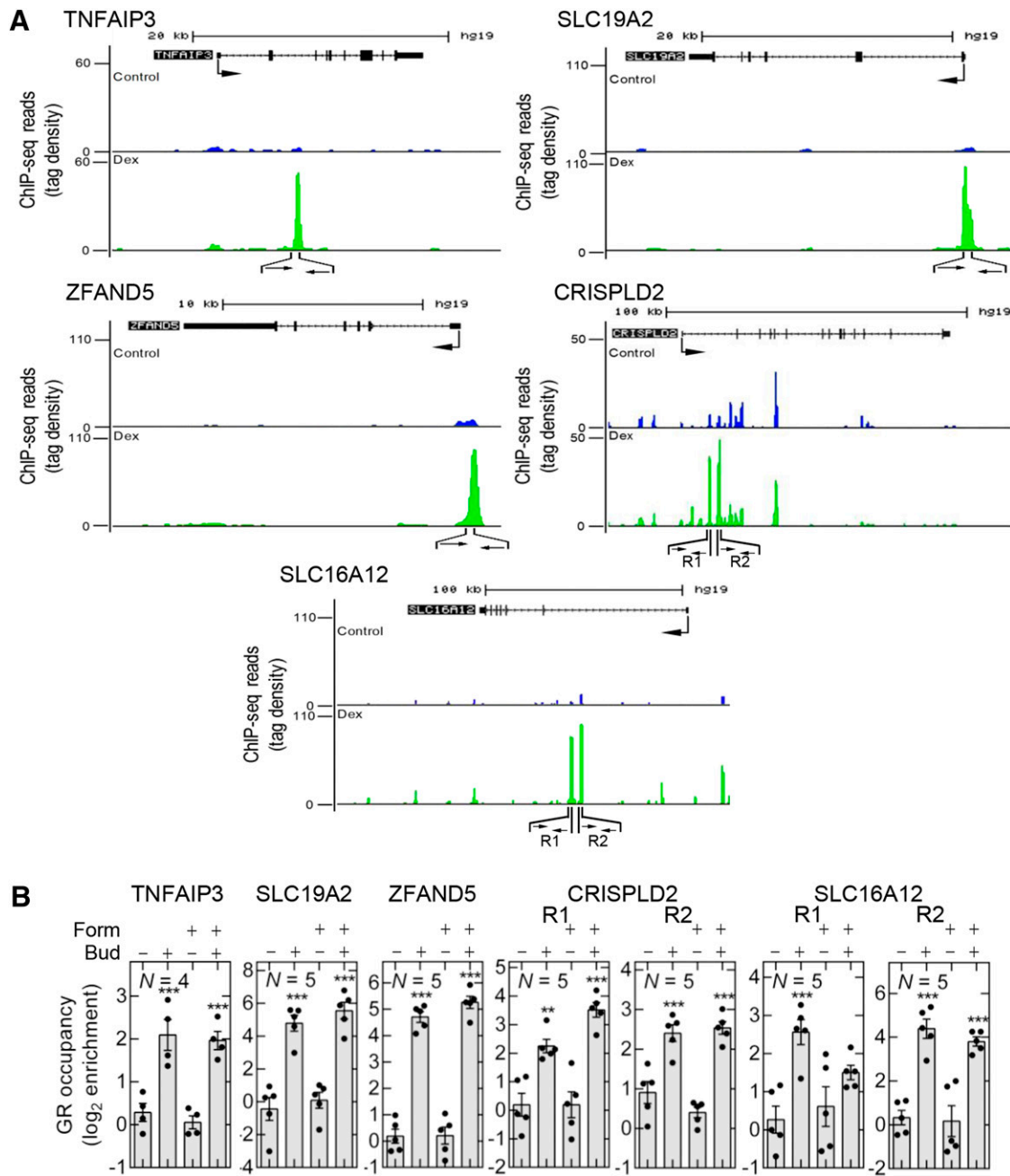


Fig. 7. Effect of LABA on GR recruitment to GR binding sites in immediate/early and delayed glucocorticoid-induced genes. (A) GR ChIP-seq analysis in control and dexamethasone (Dex; 100 nM)-treated BEAS-2B cells, published by Kadiyala et al. (2016), reveals robust GR binding sites within intronic regions of TNFAIP3, CRISPLD2, and SLC16A12 and immediately 5' to SCL19A2 and ZFAND5 (left panels). GR peak regions were checked for the existence of putative GREs using MatInspector (Genomatix) and TFBIND software. Primers for qPCR were designed, as shown, to validate single or multiple regions (R1, R2) showing robust ChIP-seq peaks. (B) BEAS-2B cells were either not stimulated or treated with formoterol (Form; 10 nM), budesonide (Bud; 100 nM), or both combined for 1 hour. GR ChIP was performed, and qPCR was performed for three unrelated control regions of genomic DNA and the indicated regions of TNFAIP3, SLC19A2, ZFAND5, CRISPLD2, and SLC16A12. Data from N experiments are shown normalized to the control regions and are plotted as log₂ enrichment (right panels). Statistical significance was tested between untreated and budesonide, formoterol and budesonide + formoterol, and between budesonide and budesonide + formoterol using analysis of variance with Bonferroni's correction. ** $P < 0.01$; *** $P < 0.001$ versus untreated. There were no significant differences between budesonide and budesonide + formoterol.

associated with the 5' region (*CDKN1C*, *KLF9*, *PDK4*, *SLC19A2*, *ZFAND5*), or both (*TSC22D3*) (Fig. 7A; Supplemental Fig. 13, B and C) (Kadiyala et al., 2016). These are consistent with direct regulation by GR, and GR recruitment was interrogated by ChIP-PCR.

Following budesonide treatment, GR was strongly recruited to GBSs at multiple loci for genes showing immediate/early

(1- to 2-hour peak) glucocorticoid-induced mRNA expression that was either markedly enhanced (TNFAIP3, SLC19A2) or unaffected by LABA (NFKBIA) (Fig. 7B; Supplemental Fig. 13B). In each case, GR binding to these sites was unaffected by formoterol alone or in combination with budesonide.

GR recruitment was also examined at GBSs associated with loci for genes showing 6-hour peak mRNA expression

that was either strongly enhanced (CRISPLD2, PDK4, ZFAND5) or modestly enhanced/unaffected (FKBP5, KLF9, KFL15, TSC22D3) by LABA. In each case, the GBSs previously identified by ChIP-seq were confirmed as showing budesonide-induced enrichment of GR (Fig. 7B; Supplemental Fig. 13C). Furthermore, formoterol alone had no effect on GR recruitment to these sites and, with the exception of the R1 binding site of CRISPLD2, did not modify budesonide-induced recruitment (Fig. 7B; Supplemental Fig. 13C). Whereas GR binding to the R2 region of CRISPLD2 was unaffected by formoterol, budesonide-induced GR binding to the R1 region was consistently enhanced by formoterol (Fig. 7B). This was borderline significant (analysis of variance using Bonferroni's correction, $P \leq 0.05$) and raises the possibility that enhanced GR recruitment could contribute toward LABA-increased expression of glucocorticoid-induced CRISPLD2.

CDKN1C mRNA is also glucocorticoid induced and synergistically enhanced by LABA. However, the CDKN1C locus is flanked by multiple genes, with the closest GR binding site lying within an intron of an adjacent gene (Supplemental Fig. 13C). Thus, a mechanistic link between GR binding and CDKN1C mRNA induction by glucocorticoid requires formal confirmation. Nevertheless, ChIP-PCR confirmed GR binding to this DNA region, and more modestly to the CDKN1C 5' promoter region, and in each case GR recruitment was unaffected by formoterol (Supplemental Fig. 13C).

Finally, SLC16A12 mRNA showed a delayed (6-hour peak) kinetic that was repressed by formoterol. Inducible GR

binding at two intronic sites was confirmed by ChIP-PCR (Fig. 7B). GR binding at the R2 region of SLC16A12 was significantly induced by budesonide and was unaffected by formoterol. Conversely, budesonide-induced GR binding to the R1 region showed a trend toward reduced recruitment in the presence of formoterol. Although not significantly changed compared with budesonide-treated, GR binding to this R1 region was also not significantly enhanced by budesonide plus formoterol when compared with untreated (Fig. 7B). Because budesonide-induced expression of SLC16A12 was reduced by formoterol, these data argue for modification of GR binding as a possible mechanism contributing to formoterol-induced repression.

LABA Enhancement of Glucocorticoid-Induced Gene Expression Can Be Insensitive to Protein Synthesis Inhibition. To investigate a requirement for protein synthesis in the ability of formoterol (10 nM) to enhance glucocorticoid-induced gene expression, qPCR was performed for selected genes following 2-hour treatment with glucocorticoid ± formoterol, each in the absence or presence of cycloheximide (Fig. 8). PTGS2 and KLF4 mRNAs both show 1- to 2-hour peak expression, and each revealed greater glucocorticoid inducibility in the presence of cycloheximide (Fig. 8A, upper panels). However, the enhancement of dexamethasone-induced PTGS2 and KLF4 mRNA expression by formoterol cotreatment was also apparent in the presence of cycloheximide (Fig. 8A, upper panels). In respect of KLF4 mRNA, formoterol enhanced dexamethasone-induced expression by ~100% (i.e., ~2-fold), and this effect was unaltered by

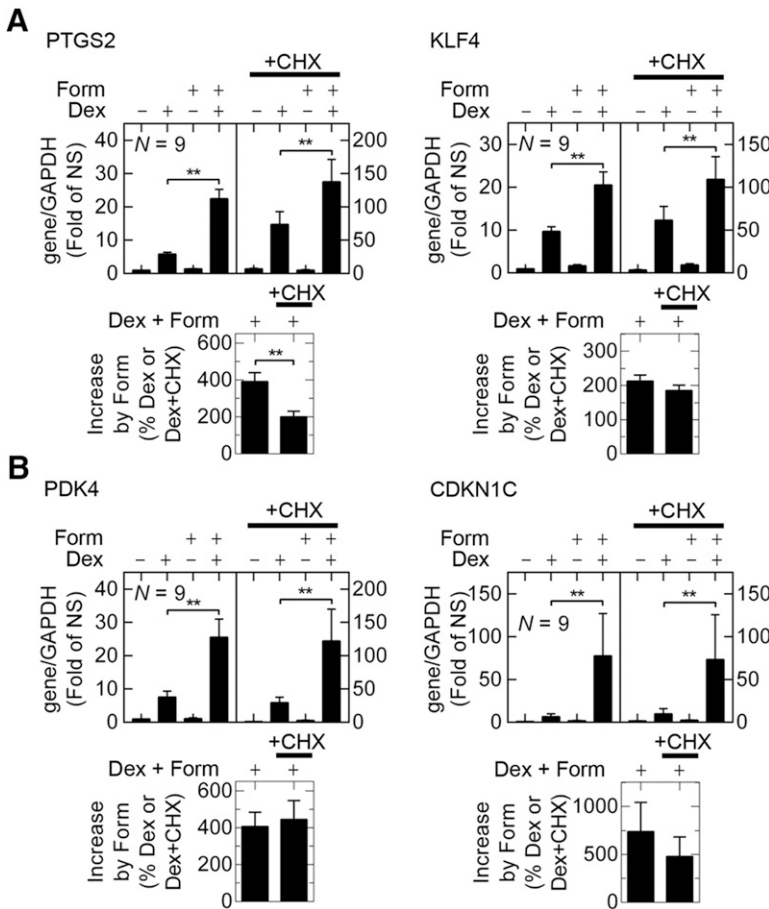


Fig. 8. Effect of the protein synthesis inhibitor, cycloheximide, on the enhancement of glucocorticoid-induced transcription by LABA for mRNAs showing the following: (A) 1- to 2-hour peaks and (B) 6-hour peaks in expression. BEAS-2B cells were either not stimulated (NS) or treated with dexamethasone (Dex; 1 μM) each in the absence or presence of formoterol (Form; 10 nM) and/or cycloheximide (CHX; 100 μg/ml), as indicated. Cells were harvested after 2 hours for qPCR analysis of the indicated genes. Data (upper panel) from *N* independent experiments, expressed as the gene of interest/glyceraldehyde-3-phosphate dehydrogenase, are plotted as fold of NS as mean ± S.E. The effect of formoterol on the induction by dexamethasone, each in the absence or presence of cycloheximide, was assessed using fold data by Wilcoxon matched-pairs signed rank test. ***P* < 0.01. Data were also replotted (lower panel) to show the effect of formoterol on the induction of luciferase RNA by dexamethasone each in the absence or presence of cycloheximide. Data are expressed as a percentage of dexamethasone or dexamethasone + cycloheximide, as appropriate, and differences in this percentage comparing without and with cycloheximide were assessed by paired *t* test. ***P* < 0.01.

cycloheximide (Fig. 8A, lower right panel). With PTGS2 mRNA, cycloheximide resulted in a 7.0 ± 1.0 -fold increase in basal expression, possibly due to the activating effect of cycloheximide on nuclear factor κ B and mitogen-activated protein kinase pathways, as previously reported (Newton et al., 1997). However, whereas formoterol cotreatment produced a significant $\sim 300\%$ (~ 4 -fold) enhancement of dexamethasone-induced PTGS2 expression, this effect was limited to ~ 2 -fold enhancement in the presence of cycloheximide (Fig. 8A, lower left panel). In the case of other mRNAs with rapid induction kinetics, KLF9, which showed a more modest, $<50\%$, enhancement of dexamethasone-induced mRNA expression by formoterol, and PLAT, which was more synergistically induced by dexamethasone plus formoterol, the enhancement produced by formoterol was essentially unaffected by cycloheximide (Supplemental Fig. 14A). However, such effects were not universal. Glucocorticoid-induced SLC19A2 mRNA was markedly enhanced by cycloheximide, but no enhancement was then observed with formoterol cotreatment (Supplemental Fig. 14A).

Analysis of PDK4 and CDKN1C mRNAs with peak expression at 6 hours, both revealed inducibility by dexamethasone that was strongly enhanced, 4- or 7-fold, respectively, by formoterol (Fig. 8B). In the case of PDK4 mRNA, whereas cycloheximide increased the inducibility by dexamethasone, the 4-fold enhancement produced by formoterol coadministration was unaltered (Fig. 8B, left panels). For CDKN1C, there was little effect of cycloheximide on dexamethasone-induced mRNA expression, and the enhancement of this by formoterol was also unaffected by cycloheximide (Fig. 8B, lower right panel). Likewise, TSC22D3 showed peak mRNA expression at 6 hours. At 2 hours, TSC22D3 mRNA was highly induced by dexamethasone and, consistent with prior data, showed a trend toward enhancement by formoterol. In the presence of cycloheximide, although glucocorticoid inducibility was markedly increased, the enhancement of this by formoterol was still apparent (Supplemental Fig. 14B). Similarly, glucocorticoid plus LABA synergy in respect of ZFAND5 persisted in the presence of cycloheximide (Supplemental Fig. 14B).

Discussion

ICS/LABA combination therapy is central to moderate-to-severe asthma management in patients where ICS monotherapy fails to achieve disease control. Accordingly, elucidating mechanisms by which ICS and LABA interact should aid efforts to rationally improve the clinical efficacy of this cost-effective therapy. Although structural and infiltrating cells are potential targets of ICS/LABA therapy, the airway epithelium is an indispensable site of glucocorticoid action (Klaßen et al., 2017). LABAs also act on the epithelium and enhance glucocorticoid-induced gene expression in both bronchial epithelial BEAS-2B cells and pHBECs (Newton and Giembycz, 2016). For this reason, BEAS-2B cells were selected to interrogate this therapeutically relevant interaction.

In BEAS-2B cells, glucocorticoids activate a luciferase reporter driven by two GRE sites, and this is enhanced by β_2 -adrenoceptor agonists (Kaur et al., 2008; Joshi et al., 2015b). In the present study, we confirm that these effects require GR and establish that the most productive interaction between LABA and glucocorticoid required concurrent addition. This is consistent with rapid-onset enhancement of

glucocorticoid-induced luciferase accumulation and the transient glucocorticoid induction of luciferase mRNA. Because increased transcription precedes luciferase mRNA accumulation, these data highlight a narrow window available for LABAs to enhance GR-driven transcription. LABAs rapidly activate a classic cAMP-PKA pathway, evidenced by the appearance of phosphorylated CREB1 within 10 minutes, and PKA is necessary for enhancement of glucocorticoid-induced GRE activity (Kaur et al., 2008; Moodley et al., 2013). Thus, observed effects can occur rapidly and are mimicked by PKA overexpression (Yeagley and Quinn, 2005). The current demonstration that the enhancement of glucocorticoid-induced $2 \times$ GRE reporter activity, or the expression of multiple genes, by formoterol persisted under conditions of protein synthesis inhibition supports a direct PKA-mediated effect.

Several mechanisms could explain how LABAs enhance GR-dependent transcription [reviewed in Newton and Giembycz (2016)]. In the current study, BEAS-2B cells are used to demonstrate that neither formoterol nor salmeterol significantly affected GR protein expression or the ability of glucocorticoids to downregulate their cognate receptor, GR. Glucocorticoid-induced GR phosphorylation at Ser²⁰³, Ser²¹¹, or Ser²²⁶, which modify receptor function (Hagood et al., 2016), was also unaffected by formoterol. Similarly, there was no effect of LABAs on the affinity of budesonide for GR. This confirms the results of a similar study that examined the effect of indacaterol on dexamethasone- and fluticasone furoate-induced GRE-dependent transcription (Joshi et al., 2015b). These data are also consistent with the lack of effect of LABAs on glucocorticoid sensitivity for the induction of the $2 \times$ GRE reporter (this study) or gene expression (Kaur et al., 2008; Rider et al., 2011; Joshi et al., 2015b). GR translocation necessarily precedes gene transcription and could represent a process that is regulated by LABAs. However, in BEAS-2B cells, salmeterol and formoterol had no effect alone or in the presence of glucocorticoid on nuclear translocation by GR. Furthermore, although budesonide recruited GR to a stably transfected $2 \times$ GRE luciferase reporter, this was unaffected by formoterol. ChIP-seq data from dexamethasone-treated BEAS-2B cells identified GBSs associated with multiple loci for glucocorticoid-induced mRNAs (Kadiyala et al., 2016). These may, or may not, show modulation by LABA. For example, ChIP-PCR confirmed budesonide-induced recruitment of GR to *FKBP5* and *NFKBIA*. This was unaffected by LABA, and these mRNAs were not LABA-enhanced. ChIP-PCR also confirmed GR recruitment to 13 GBSs near to, or within, several budesonide-induced genes (*TNFAIP3*, *CRISPLD2*, *ZFAND5*, *SLC19A2*, *PDK4*, *CDKN1C*, *TSC22D3*, *KLF9*, and *KLF15*), mRNAs for which were either highly, or more modestly, enhanced by formoterol cotreatment. At 12 of these sites, budesonide-induced GR recruitment was unaffected by formoterol. At one GBS, the R1 region of *CRISPLD2*, formoterol appeared to enhance budesonide-induced GR DNA binding. Conversely, glucocorticoid-induced SLC16A12 mRNA was repressed by formoterol. GR binding to the two main intronic sites for this gene showed robust budesonide-induced GR recruitment. However, binding to one site (R2) was unaffected by formoterol cotreatment, whereas the R1 region indicated reduced GR recruitment. Clearly, these data do not support general effects upstream of GR DNA binding to account for the LABA enhancement of GR-driven transcription. This is important because LABAs do not invariably enhance

GR-dependent transcription; indeed, whereas many mRNAs were induced by glucocorticoid, only a fraction was enhanced by LABA cotreatment, and some were even repressed. Similar gene expression patterns occur in pHBECS (manuscript in preparation). Thus, mechanistic explanations invoking generic effects on GR function cannot reconcile these data. Modulation of GR expression, glucocorticoid-binding affinity, or GR nuclear translocation should affect all GR-induced genes. This was not apparent and is convincingly illustrated by reference to RU486, a glucocorticoid that usually behaves as a GR antagonist, but that shows transactivation in the presence of PKA activators (Nordeen et al., 1993). In BEAS-2B cells, RU486 induced GR translocation, but was transcriptionally silent on the 2×GRE reporter. However, LABAs showed no effect on GR translocation but, nevertheless, converted RU486 into a weak partial agonist. Conversely, GR recruitment to the 2×GRE reporter and many glucocorticoid-induced gene loci was unaffected by LABA. Thus, the enhancement of GR-dependent transcription in BEAS-2B cells, and most likely in pHBECS, by LABAs must rely on pathways that modify transcriptional activation of specific genes rather than generic events leading to GR DNA binding.

Although LABAs and glucocorticoids independently modulate gene expression, the gene-specific nature of synergy warrants comment. On the 2×GRE reporter, LABAs were inactive, yet they amplified glucocorticoid-induced transactivation without changing GR recruitment. This may be explained by the ability of the catalytic subunits of PKA to migrate to the nucleus (Meja et al., 2004) and phosphorylate substrates that promote GR-dependent transcription in a gene-dependent manner. Indeed, coactivators, basal and other components of the transcriptional machinery, histones, chromatin-modifying proteins, and RNA polymerase II are all regulated phospho-proteins (Rowan et al., 2000; Vo and Goodman, 2001; Johannessen et al., 2004; Brookes and Pombo, 2009; Badeaux and Shi, 2013). Furthermore, many, including CREB1-binding protein and histones, are PKA targets (Vo and Goodman, 2001; Badeaux and Shi, 2013). Likewise, GR phosphorylation (other than at Ser²⁰³, Ser²¹¹, or Ser²²⁶) could produce gene-specific effects by modifying promoter-specific interactions.

Direct modulation of GR-dependent transcription is unlikely as a sole mechanism for LABA enhancement. Glucocorticoid-induced genes showing LABA enhancement were either induced by budesonide (≥ 2 -fold, $P \leq 0.05$), but not formoterol (< 2 -fold), or were induced by both drugs (each ≥ 2 -fold, $P \leq 0.05$). For mRNAs showing independent LABA inducibility, one can presume roles for cAMP-activated transcription factors, such as CREB1 or related family members (Johannessen et al., 2004; Sands and Palmer, 2008). For example, CREB1 activates multiple genes, including *NR4A2*, which is highly upregulated by LABA in BEAS-2B cells (Zhang et al., 2005; Yan et al., 2018). Similarly, CAAT/enhancer box proteins are activated and/or induced by cAMP (Croniger et al., 1998; Ramji and Foka, 2002). These factors induce cAMP-dependent transcription and can participate in coregulation of transcription with GR (Croniger et al., 1998; Ramji and Foka, 2002; Roos and Nord, 2012). Thus, transcriptional activation by GR and cAMP-activated factors represents a plausible explanation for glucocorticoid/LABA synergy at mRNAs, for example,

SLC19A2 and *CRISPLD2*, which are independently glucocorticoid- and LABA-inducible. Likewise, many budesonide-induced, formoterol-enhanced genes, for which formoterol inducibility was < 2 -fold, were, nevertheless, significantly induced by formoterol alone. Thus, budesonide-induced expression of *TNFAIP3* and *PLAT* was enhanced by formoterol cotreatment, and these mRNAs were modestly upregulated by formoterol alone. Similarly, whereas *PDK4* and *CDKN1C* mRNAs were not significantly formoterol-induced in the current microarray data, LABA-induced increases were apparent by qPCR (Joshi et al., 2015b). Thus, induction by LABA is not readily separable from the enhancement of glucocorticoid-dependent effects and implies that independent activation by GR and cAMP-induced transcription factors could readily account for synergy. Independent LABA inducibility was most apparent for those genes that were rapidly induced by glucocorticoid (1- to 2-hour peak) when compared with more slowly induced mRNAs. This is consistent with early induction kinetics for most LABA-induced genes, whereas transcription factors induced early could drive later-onset synergies. However, minimal, or even a lack of, induction by one stimulus is not incompatible with this model. Binding, or activation, by one factor may be necessary for binding, or activation, of another. For example, CAAT/enhancer box proteins promote chromatin rearrangements that can improve access and transcriptional activation by a second factor, such as GR (Grøntved et al., 2013). Although the current data do not support a general LABA enhancement of GR binding to DNA, this is not excluded at specific sites. Indeed, such effects could underlie the apparent LABA-enhanced recruitment of GR to the *CRISPLD2* R1 region.

In summary, LABAs do not enhance expression of all glucocorticoid-inducible genes; their effects are gene-specific. Therefore, understanding the function of individual genes is necessary to appreciate those aspects of asthma pathogenesis that will respond most effectively to ICS/LABA therapy. Furthermore, knowledge of gene function is critical to optimize therapy. Knowing which glucocorticoid-inducible genes are beneficial, or undesirable, will aid screening strategies to identify novel GR ligands (steroidal or nonsteroidal) that most effectively capture desirable synergies with a LABA. This may be especially important in severe asthma, in which new GR agonists may be combined with a LABA to offer cost-effective alternatives to immune-modulatory biologicals.

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Authorship Contributions

Participated in research design: Rider, Kärrman-Mårdh, Miller-Larsson, Gerber, Giembycz, Newton.

Conducted experiments: Rider, Altonsy, Mostafa, Shah, Sasse, Manson, Yan, Newton.

Contributed new reagents or analytic tools: Gerber.

Performed data analysis: Rider, Altonsy, Mostafa, Shah, Sasse, Manson, Yan, Gerber, Newton.

Wrote or contributed to the writing of the manuscript: Rider, Altonsy, Mostafa, Shah, Sasse, Manson, Yan, Kärrman-Mårdh, Miller-Larsson, Gerber, Giembycz, Newton.

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