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Inflammatory Stimuli Inhibit Glucocorticoid-Dependent Transactivation in Human Pulmonary Epithelial Cells: Rescue by Long-Acting β_2-Adrenoceptor Agonists[S]

Christopher F. Rider, Elizabeth M. King, Neil S. Holden, Mark A. Giembycz, and Robert Newton

Airways Inflammation Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

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ABSTRACT

By repressing inflammatory gene expression, glucocorticoids are the most effective treatment for chronic inflammatory diseases such as asthma. However, in some patients with severe disease, or who smoke or suffer from chronic obstructive pulmonary disease, glucocorticoids are poorly effective. Although many investigators focus on defects in the repression of inflammatory gene expression, glucocorticoids also induce (transactivate) the expression of numerous genes to elicit anti-inflammatory effects. Using human bronchial epithelial (BEAS-2B) and pulmonary (A549) cells, we show that cytokines [tumor necrosis factor α (TNFα) and interleukin 1β], mitogens [fetal calf serum (FCS) and phorbol ester], cigarette smoke, and a G_q-linked G protein-coupled receptor agonist attenuate simple glucocorticoid response element (GRE)-dependent transcription. With TNFα and FCS, this effect was not overcome by increasing concentrations of dexamethasone, budesonide, or fluticasone propionate. Thus, the maximal ability of the glucocorticoid to promote GRE-dependent transcription was reduced, and this was shown additionally for the glucocorticoid-induced gene *p57^{KIP2}*. The long-acting β_2-adrenoceptor agonists (LABAs) formoterol fumarate and salmeterol xinafoate enhanced simple GRE-dependent transcription to a level that could not be achieved by glucocorticoid alone. In the presence of TNFα or FCS, which repressed glucocorticoid responsiveness, these LABAs restored glucocorticoid-dependent transcription to levels that were achieved by glucocorticoid alone. Given the existence of genes, such as *p57^{KIP2}*, which may mediate anti-inflammatory actions of glucocorticoids, we propose that repression of transactivation represents a mechanism for glucocorticoid resistance and for understanding the clinical benefit of LABAs as an add-on therapy in asthma and chronic obstructive pulmonary disease.

Introduction

Asthma is a chronic disease characterized by nonspecific airway hyperresponsiveness, reversible bronchoconstriction, airway remodeling, and inflammation, which may include both eosinophilic and, particularly in severe asthma, neutrophilic components (Jatakanon et al., 1999; Louis et al., 2000). Currently, the most effective treatments for all but the mildest asthmatic patients are glucocorticoids, which are taken in an inhaled form and are known clinically as inhaled corticosteroids (ICSs) (Barnes, 2006). By down-regulating inflammatory gene expression, ICSs reduce lung inflammation to improve symptoms and reduce exacerbation frequency (Barnes, 2006). Although ICSs control symptoms in the majority of asthmatic patients, 25 to 35% may show reduced or suboptimal responsiveness to therapy (Mjaanes et al., 2006; Zeiger et al., 2006; Martin et al., 2007). In this respect, it appears that the nature of the inflammation may play a determining role. Thus, glucocorticoid sensitivity is reduced

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ABBREVIATIONS: ICS, inhaled corticosteroid; ANOVA, analysis of variance; AP-1, activator protein 1; Bud, budesonide; CSE, cigarette smoke extract; Dex, dexamethasone; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; Form, formoterol; FP, fluticasone propionate; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HDAC, histone deacetylase; IL, interleukin; JNK, c-Jun N-terminal kinase; LABA, long-acting β_2-adrenoceptor agonist; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PCR, polymerase chain reaction; U46619, 9,11-dideoxy-11a,9a-epoxymethano-prostaglandin $F_{2\alpha}$; PMA, phorbol 12-myristate 13-acetate; Salm, salmeterol; TNFα, tumor necrosis factor α.

in patients with severe disease who often show predominantly neutrophilic airway inflammation, whereas individuals with eosinophilic asthma are generally glucocorticoid responsive (Barnes and Adcock, 2009; Newton et al., 2010b). In addition, asthmatic patients who smoke or individuals who suffer from chronic obstructive pulmonary disease, a smoking-induced condition that is characterized by an irreversible decline in lung function, typically show poor responsiveness to glucocorticoids (Braganza et al., 2008). Although there are multiple reasons for this, which may include genetic factors, it is equally clear that proinflammatory stimuli reduce responsiveness to glucocorticoids (Barnes and Adcock, 2009; Newton et al., 2010b). Thus, there is an unmet need to understand and improve glucocorticoid efficacy in these patient groups.

Binding of glucocorticoid to the glucocorticoid receptor (GR) (NR3C1) induces translocation of the receptor from the cytoplasm to the nucleus, where GR modulates gene transcription in both positive and negative fashions (Newton and Holden, 2007). Thus, GR interacts with proinflammatory transcription factor complexes, such as nuclear factor κB or activator protein 1 (AP-1), to recruit histone deacetylases (HDACs) and decrease the transcription of inflammatory genes (an effect referred to as transrepression) (Barnes, 2006). In contrast, GR induces transcriptional responses from a variety of simple and complex glucocorticoid response elements (GREs) (Newton and Holden, 2007). Despite this, the key anti-inflammatory activities of glucocorticoids are attributed generally to direct transrepression by GR (Barnes, 2006). However, in the context of inflammation, proinflammatory cytokines, for example, tumor necrosis factor α (TNFα), may attenuate the ability of glucocorticoids to inhibit proinflammatory transcription factor activity by both increasing activation of c-Jun N-terminal kinase (JNK) and other mitogen-activated protein kinases (MAPKs) and reducing the expression of HDAC2 (Barnes and Adcock, 2009). In contrast, the repressive effect of glucocorticoids acting on core inflammatory genes, including cyclooxygenase 2, interleukin (IL)-6, IL-8, and granulocyte/macrophage colony-stimulating factor, can be blocked by inhibitors of transcription or translation (Newton and Holden, 2007; Newton et al., 2010a). Such data imply that glucocorticoid-dependent repression of inflammatory genes involves the expression of glucocorticoid-induced genes (Newton and Holden, 2007). Certainly, glucocorticoids induce the expression of many hundreds of target genes, and many of these display properties that are consistent with anti-inflammatory effects (Newton and Holden, 2007). Thus MAPK phosphatase 1, also called dual-specificity phosphatase 1, is induced profoundly and rapidly by glucocorticoids to switch off MAPK pathways, reduce inflammatory gene transcription, and destabilize inflammatory gene mRNAs (Issa et al., 2007; Clark et al., 2008; Diefenbacher et al., 2008; King et al., 2009; Newton et al., 2010a). Likewise, glucocorticoid-induced leucine zipper (GILZ) (TSC22D3) mRNA and protein are induced highly by glucocorticoids and act to reduce transcriptional responses via factors including AP-1 and nuclear factor κB (Mittelstadt and Ashwell, 2001; Eddleston et al., 2007). Another protein with putative anti-inflammatory properties is cyclin-dependent kinase inhibitor 1c, also known as $p57^{KIP2}$, which regulates cell cycle progression and can switch off the JNK/MAPK pathway (Samuelsson et al., 1999; Chang et al., 2003).

Taken together, it is increasingly apparent that transactivation by GR contributes toward the anti-inflammatory activity of glucocorticoids. Indeed, although some patterns of steroid resistance may reflect altered GR translocation, a number of patients show changes in histone modification that suggest a failure of GR transactivation rather than transrepression (Matthews et al., 2004). Given this, it is plausible that transactivation of anti-inflammatory genes also may be the subject of glucocorticoid resistance. In the current study, our objective was to determine whether glucocorticoid-induced gene expression was reduced by inflammatory stimuli. Consequently, we have investigated the effects of various stimuli on the ability of glucocorticoids to induce transcription from a simple GRE-dependent reporter and on the expression of two glucocorticoid-inducible genes, *$p57^{KIP2}$* and *GILZ*, that have anti-inflammatory potential.

Materials and Methods

Cell Culture and Drugs. Type II epithelial (A549) and bronchial epithelial (BEAS-2B) cells were cultured to confluence in Dulbecco's modified Eagle's medium or Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA) with 14 mM $NaHCO_3$ added, respectively, supplemented with 10% fetal calf serum (FCS) (Canadian sourced; Invitrogen) and 2 mM L-glutamine. Before all of the experiments, cells were incubated overnight in serum-free medium before replacing with fresh serum-free medium containing drugs and cytokines. Dexamethasone was dissolved in Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO). Budesonide, formoterol furoate (formoterol), fluticasone propionate, salmeterol xinafoate (salmeterol) (Sigma-Aldrich), 9,11-dideoxy-11a,9a-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) (Cayman Chemical, Ann Arbor, MI), and phorbol 12-myristate 13-acetate (PMA) (Enzo Life Sciences, Farmingdale, NY) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) (final DMSO concentration <0.1%). Recombinant human IL-1β and TNFα (R&D Systems, Minneapolis, MN) were dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin. H_2O_2 (Sigma-Aldrich) and FCS (Invitrogen) were diluted in serum-free medium before experiments. Cigarette smoke extract-conditioned medium (CSE) was prepared by bubbling the smoke from one research-grade cigarette (3R4F 12/2006; University of Kentucky, Lexington, KY) through 4 ml of serum-free medium (Hudy et al., 2010). Optical density at 320 nm (OD_{320}) was measured, and fresh medium added to give an initial OD_{320} of 1.

Reporter Constructs and Luciferase Assay. A549 and BEAS-2B cells stably transfected with the 2×GRE-containing luciferase reporter, pGL3.neo.TATA.2GRE (Chivers et al., 2006), were cultured to confluence in 24-well plates. Luciferase assays were performed according to the manufacturer's guidelines (Firefly Luciferase Assay Kit; Biotium, Hayward, CA).

Cell Viability Assay. Cells in 24-well plates were analyzed for cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After aspiration, MTT solution (1 mg/ml in phosphate-buffered saline) (200 μl/well) was added for 30 min at 37°C. Wells were aspirated, and DMSO (200 μl) was added before the measurement of absorbance at 584 nm.

Calcium Assay. Confluent BEAS-2B cells were loaded for 2 h with Fluo-4 (Fluo-4 NW Calcium Assay Kit; Invitrogen) at 37°C, according to the manufacturer's guidelines. With excitation at 494 nm, basal fluorescence (F_0) was measured at 516 nm for 1.6 s in a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany). The thromboxane receptor agonist U46619 (Cayman Chemical) was injected, and fluorescence was measured for a further 48.4 s. Values were expressed as F/F_0, which is a proxy for intracellular calcium concentration ($[Ca^{2+}]_i$).

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction. Total RNA was extracted from cells in 12-well plates using the RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA was prepared from 0.5 μg of RNA using a qScript cDNA synthesis kit (Quanta, Gaithersburg, MD) before being diluted 1:5 in DNase-free water. Real-time polymerase chain reaction (PCR) was performed using a 7900HT instrument (Applied Biosystems Inc., Foster City, CA) on 2.5 μl of cDNA in a reaction volume of 10 μl using SYBR Green chemistry (Invitrogen). Amplification conditions of 50°C, 2 min; 95°C, 10 min; then 40 cycles of 95°C, 15 s; 60°C, 1 min were used before dissociation (melt curve) analysis to confirm primer specificity (King et al., 2009). PCR primers were: GILZ (TSC22D3), forward 5′-GGC CAT AGA CAA CAA GAT CG-3′, reverse 5′-ACT TAC ACC GCA GAA CCA CCA-3′; cyclin-dependent kinase inhibitor 1c/p57^{KIP2}, forward 5′-CGG CGA TCA AGA AGC TGT C-3′, reverse 5′-GGC TCT AAA TTG GCT CAC CG-3′; and glyceraldehyde 3-phosphate dehydrogenase, forward 5′-TTC ACC ACC ATG GAG AAG GC-3′, reverse 5′-AGG AGG CAT TGC TGA TGA TCT-3′.

Western Blot Analysis. Cells were lysed in 1× Laemmli buffer supplemented with 1× complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Sigma-Aldrich). Total cell lysates were size-fractionated by Tris-glycine SDS polyacrylamide gel electrophoresis on 10 or 15% gels before electrotransfer to Hybond ECL membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Membranes were blocked in 5% milk/phosphate-buffered saline before being probed with GILZ antiserum (provided by Bruce Zuraw) (Eddleston et al., 2007), cyclin-dependent kinase inhibitor 1c/p57^{KIP2} (2557; Cell Signaling Technology, Danvers, MA), or glyceraldehyde 3-phosphate dehydrogenase (4699–9555(ST); AbD Serotec, Raleigh, NC). After incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig, immune complexes were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA). Densitometry was performed on Western blot images acquired below the saturation level of the film, using TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Statistical Analysis. Data are presented as means ± S.E. Statistical analysis was performed in GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA), using repeated measures one-way analysis of variance (ANOVA) with Bonferroni's or Dunnett's post-tests or paired *t* tests as appropriate.

Results

Effects of TNFα and FCS on Simple GRE-Dependent Transcription. BEAS-2B cells that had been transfected stably with the 2×GRE luciferase reporter, pGL3.neo.TATA.2GRE were treated with 10 ng/ml TNFα for various periods, both before and after stimulation with a maximally effective concentration (1 μM) of the synthetic glucocorticoid dexamethasone (Kaur et al., 2008; King et al., 2009) (Fig. 1A). Dexamethasone induced simple GRE-dependent transcription, and this was significantly and maximally reduced to 54% of the dexamethasone-induced response with a 1-h preincubation with TNFα (Fig. 1A). Near maximal inhibition was observed with 2- and 0.5-h preincubations with TNFα (Fig. 1A). This effect was concentration-dependent, with maximal repression being achieved at a TNFα concentration of 10 ng/ml (Fig. 1B). To examine whether the effect was generic or simply specific to BEAS-2B cells, the effects of both TNFα and IL-1β were tested on the pGL3.neo.TATA.2GRE reporter in A549 type II pulmonary cells (Fig. 1, C and D). In each case, dexamethasone-induced luciferase activity was repressed significantly and progressively by increasing concentrations of TNFα or IL-1β.

Serum is an established mitogen and activates many kinase cascades including MAPKs. BEAS-2B cells harboring the 2×GRE reporter therefore were treated with 10% FCS for various times before or after stimulation with 1 μM dexamethasone (Fig. 2A). As with TNFα, preincubation with 10% FCS for 1 and 2 h significantly reduced 2×GRE reporter activity. This was not apparent with a 6-h preincubation but was again apparent after an 18-h preincubation with FCS. With a 1-h preincubation, the effect of FCS was found to be concentration dependent and occurred in both BEAS-2B and A549 cells (Fig. 2, B and C). Because FCS is a complex mixture of growth factors, hormones, and other biological entities, BEAS-2B cells were treated with epidermal growth factor to determine whether this alone was able to induce glucocorticoid resistance. However, despite activation of the JNK pathway being induced, there was no obvious effect of epidermal growth factor on simple 2×GRE-dependent transcription induced by dexamethasone (Supplemental Fig. 1).

Because proinflammatory cytokines, such as TNFα, may induce different signaling pathways to FCS, we tested whether these two agents could combine to yield a greater overall repression (Fig. 2D). As above, 1-h preincubation with TNFα resulted in a concentration-dependent reduction in dexamethasone-induced GRE-dependent transcription. Like-

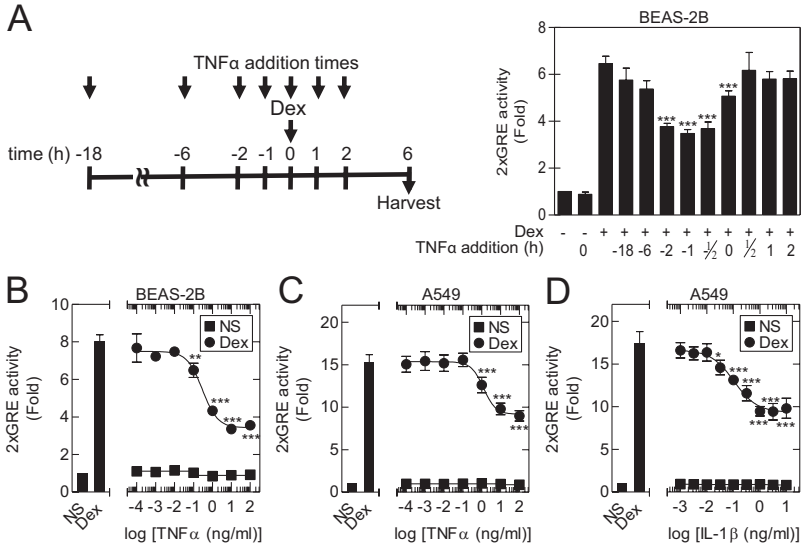

Fig. 1. Effects of proinflammatory cytokines on simple GRE-dependent transcription. A, BEAS-2B cells harboring a 2×GRE reporter were treated with TNFα (10 ng/ml) for up to 18 h before and up to 2 h after the addition of dexamethasone (1 μM) (Dex), as depicted (left panel). Cells were harvested for luciferase assay 6 h after the addition of Dex. Data ($n = 6$), expressed as fold activation, are plotted as means ± S.E. B to D, BEAS-2B cells (B) or A549 cells (C and D) harboring a 2×GRE-dependent luciferase reporter were pretreated for 1 h, or not, with various concentrations of TNFα (B and C) or IL-1β (D) before either no stimulation (NS) or treatment with Dex (1 μM). Cells were harvested 6 h after the addition of Dex for luciferase assay. Data ($n = 4$, 6, and 4, respectively), expressed as fold activation, are plotted as means ± S.E. Statistical analyses, versus Dex control, were performed by ANOVA with a Dunnett's post-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

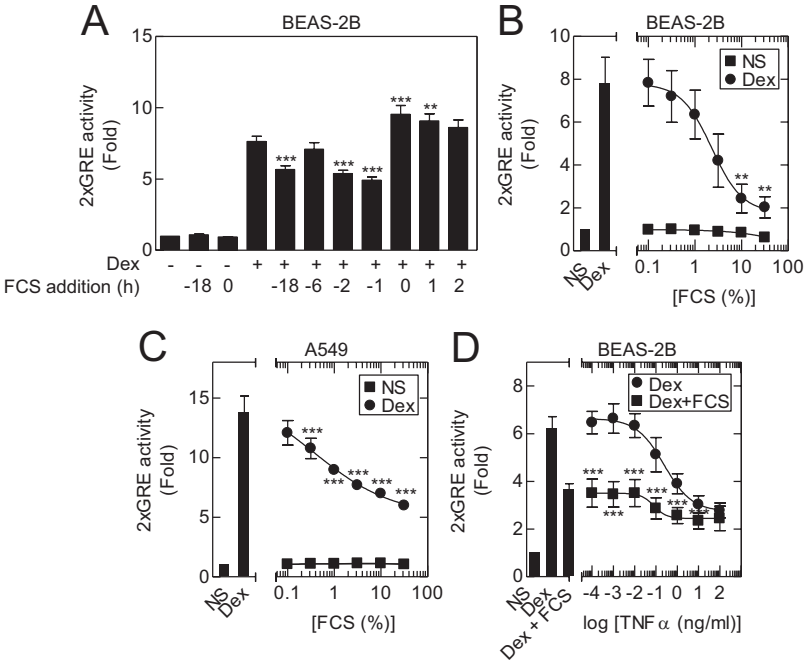

Fig. 2. Effects of FCS on simple GRE-dependent transcription. A, BEAS-2B cells harboring a 2×GRE reporter were treated with 10% FCS for up to 18 h before and up to 2 h after the addition of dexamethasone (1 μM) (Dex). Cells were harvested for luciferase assay 6 h after the addition of Dex. Data (n = 8), expressed as fold activation, are plotted as means ± S.E. B and C, BEAS-2B cells (B) or A549 cells (C) harboring a 2×GRE-dependent luciferase reporter were pretreated for 1 h, or not, with various concentrations of FCS before treatment with Dex (1 μM). Cells were harvested after 6 h for luciferase assay. Data (n = 6), expressed as fold activation, are plotted as means ± S.E. D, BEAS-2B cells harboring a 2×GRE reporter were pretreated for 1 h, or not, with 10% FCS and various concentrations of TNFα, before the addition of Dex (1 μM). Cells were harvested for luciferase assay 6 h after Dex addition. Data (n = 8), expressed as fold activation, are plotted as means ± S.E. A to C, statistical analyses, versus Dex control, were performed by ANOVA with a Dunnett's post-test. D, differences in the repression produced by different concentrations of TNFα, in the absence or presence of FCS, were examined by paired t test. **, $P < 0.01$; ***, $P < 0.001$.

wise, 10% FCS produced a 40% drop in dexamethasone-induced luciferase activity, which in the presence of TNFα further declined to the level that was achieved by the highest concentrations of TNFα. These data do not support the concept of separate pathways of repression but suggest that both TNFα and FCS may act on a common pathway, which once maximally activated cannot produce greater repression of GRE-dependent transcription.

It is possible that increasing the concentration of glucocorticoid may overcome the repressive effect that is exerted by TNFα or FCS. Therefore, BEAS-2B 2×GRE reporter cells were pretreated with either TNFα (10 ng/ml) or FCS (10%) before stimulation with various concentrations of dexamethasone. Dexamethasone produced maximal activation of the reporter at a concentration of approximately 1 μM with EC_{50} values in the 26 to 50 nM range (Fig. 3A; Supplemental Table 1). Although this is consistent with previous data (Kaur et al., 2008), the presence of TNFα or FCS significantly reduced the maximal response to dexamethasone, without significantly altering the EC_{50} value (Fig. 3A; Supplemental Table 1). Qualitatively similar data were obtained with respect to budesonide and fluticasone propionate (Fig. 3A; Supplemental Table 1). Likewise, the repression of GRE-dependent transcription that was exerted by TNFα, IL-1β, and FCS in A549 cells also was not overcome by elevated concentrations of dexamethasone (Fig. 3). However, both TNFα and FCS modestly increased (by 35 and 28%, respectively) the EC_{50} values for dexamethasone (Fig. 3; Supplemental Table 1). Thus, the repressive effect that occurs with all of the glucocorticoids is common to at least two different cell lines and is not surmountable with increased concentrations of glucocorticoid.

Effect of Protein Kinase C Activation and Signaling from a G_q-Linked G Protein-Coupled Receptor. Mediators, including histamine, leukotrienes, and prostanoids such as prostaglandin D_2 and thromboxane, are released from multiple cell types and can elicit bronchospasm (Barnes et al., 1998). Such agonists act on G protein-coupled receptors that couple via the G protein $G_q\alpha$ to phospholipase C. This results in hydrolysis of phosphatidylinositol (4,5)-bisphosphate to yield inositol (1,4,5)-trisphosphate and diacylglycerol, which promotes intracellular calcium release ($[Ca^{2+}]_i$) and activates protein kinase C, respectively. BEAS-2B 2×GRE reporter cells therefore were pretreated with a maximally effective concentration (100 nM) of the protein kinase C activator PMA (Fig. 4A). Both 1- and 2-h pretreatments with PMA resulted in significant reductions in dexamethasone-induced 2×GRE reporter activity. This effect was concentration dependent, with the maximum observed repression being achieved at 1 μM PMA (Fig. 4B).

To test whether a bona fide G protein-coupled receptor agonist could elicit a similar effect, BEAS-2B cells were treated with various concentrations of the thromboxane analog U46619. Effects on $[Ca^{2+}]_i$ were monitored using the calcium-sensing dye Fluo-4, which revealed a maximal response in the presence of 1 μM U46619 that occurred within seconds of adding the drug (Fig. 4C). However, 1-h pretreatment of BEAS-2B cells harboring the 2×GRE reporter revealed no effect of U46619 on dexamethasone-induced luciferase activity (Fig. 4D). In contrast, preincubations of 2, 6, and 18 h with U46619 produced significant repression of GRE-dependent transcription induced by dexamethasone (Fig. 4E). Likewise, the acetylcholine receptor agonist carbachol robustly induced $[Ca^{2+}]_i$ in BEAS-2B cells with a maximally effective concentration of 100 μM (Supplemental Fig. 2A). However, no significant effects on dexamethasone-induced GRE-dependent transcription were elicited at any time tested (Supplemental Fig. 2B).

Effects of CSE and H_2O_2 on Simple GRE-Dependent Transcription. CSE, diluted to different OD_{320} values in serum-free medium, was added to both BEAS-2B and A549 cells harboring the 2×GRE reporter (Fig. 5, A and B). In each case, dexamethasone-induced reporter activity was reduced significantly in a concentration-dependent fashion. As determined by the MTT assay, these treatments resulted in no significant effect on cell viability at CSE concentrations of

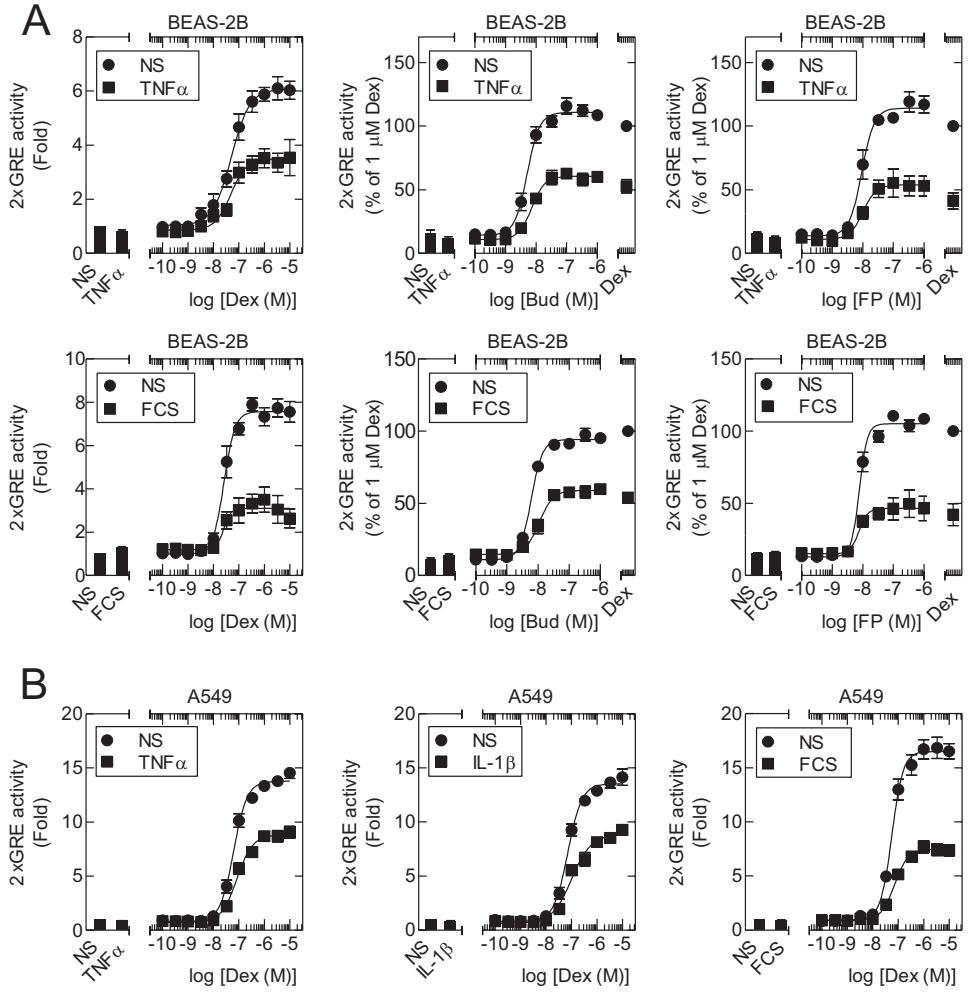

Fig. 3. Repression of simple GRE-dependent transcription by TNFα or FCS is not overcome by high-concentration glucocorticoid. A, BEAS-2B cells harboring a 2×GRE-dependent luciferase reporter were pretreated for 1 h, or not, with TNFα (10 ng/ml) or 10% FCS before treatment with various concentrations of dexamethasone (Dex), fluticasone propionate (FP), or budesonide (Bud). Where FP or Bud were used, the effect of Dex (1 μM) also was tested. Cells were harvested after 6 h for luciferase assay. Data (n = 4–6), expressed as fold activation or as a percentage of 1 μM Dex, are plotted as means ± S.E. Induction in the absence and presence of TNFα or FCS was compared by paired t test. The effect of each concentration of glucocorticoid over 10 nM was repressed significantly (to at least $P < 0.05$) by TNFα or FCS (asterisks not shown). B, A549 cells harboring a 2×GRE-dependent luciferase reporter were pretreated for 1 h, or not, with TNFα (10 ng/ml), IL-1β (1 ng/ml), or 10% FCS before Dex (1 μM) addition. Cells were harvested after 6 h for luciferase assay. Data (n = 6–8), expressed as fold activation, are plotted as means ± S.E. Induction in the absence and presence of TNFα, IL-1β, or FCS was compared by paired t test. The effect of each concentration of glucocorticoid at or over 10 nM was repressed significantly (to at least $P < 0.05$) by TNFα, IL-1β, or FCS (asterisks not shown).

OD 0.3 or below in BEAS-2B cells but produced minimal effects at OD 0.3 in A549 cells and some loss of cell viability at the highest concentration tested (OD 1.0) in both cell lines. Thus, although high concentrations of CSE are clearly toxic to cells, the ability to inhibit GRE-dependent transcription occurs at lower, nontoxic concentrations of CSE. In contrast, whereas the addition of H_2O_2 to BEAS-2B cells also resulted in significant repression of dexamethasone-induced reporter activity, this closely correlated with a substantial loss in cell viability (Fig. 5C). Thus, any repressive effects simply may be secondary to cell death.

Rescue of Repression by Long-Acting β_2-Adrenoceptor Agonists. In asthmatic patients whose symptoms are controlled poorly by inhaled glucocorticoids, the addition of a LABA is recommended as a step-up treatment option (Barnes, 2006). LABAs enhance the anti-inflammatory effect of the inhaled glucocorticoid to a level that cannot be achieved by the glucocorticoid alone (Giembycz et al., 2008; Newton et al., 2010b). Accordingly, we assessed the effects of LABAs on TNFα- and FCS-induced resistance to glucocorticoids. BEAS-2B cells harboring the simple 2×GRE reporter were pretreated with TNFα or FCS before stimulation with dexamethasone in the absence or presence of the LABAs salmeterol and formoterol (Fig. 6). As described under *Effects of CSE and H_2O_2 on Simple GRE-Dependent Transcription*, dexamethasone induced 2×GRE activity, and this was reduced significantly by TNFα or FCS preincubation (Fig. 6; Tables 1 and 2). In the presence of dexamethasone, increasing concentrations of salmeterol or formoterol progressively increased reporter activity to approximately three times the level achieved by dexamethasone alone. In the presence of either FCS or TNFα, which significantly repressed dexamethasone-induced 2×GRE activity, both salmeterol and formoterol increased dexamethasone-induced 2×GRE activity back to or slightly above the level that was achieved by dexamethasone alone (Fig. 6; Tables 1 and 2). Thus, LABAs restored the level of GRE-dependent transcription to that seen in the absence of proinflammatory or mitogenic stimuli. In each case, the sensitivity to the LABAs was unaltered, with EC_{50} values for the enhancement of 2×GRE-dependent transcription in the absence or presence of 10% FCS of: salmeterol, 5.99 ± 1.21 × 10^{-10} M (no FCS) and 7.69 ± 1.73 × 10^{-10} M (+FCS); formoterol 7.30 ± 1.21 × 10^{-11} M (no FCS) and 8.32 ± 1.86 × 10^{-11} M (+FCS). Likewise, EC_{50} values for the enhancement of 2×GRE-dependent transcription in the absence and presence of TNFα (10 ng/ml) were: salmeterol, 9.58 ± 1.20 × 10^{-10} (no TNFα), 8.54 ± 1.22 × 10^{-10} (+TNFα); formoterol, 6.06 ± 1.16 × 10^{-11} (no TNFα), 5.93 ± 1.56 × 10^{-11} (+TNFα). Despite the unaltered sensitivity, it is apparent that the overall enhancement of the response to dexamethasone that was achieved by the LABAs in the presence of FCS or TNFα was less than that achieved in the absence of FCS or TNFα (Tables 1 and 2).

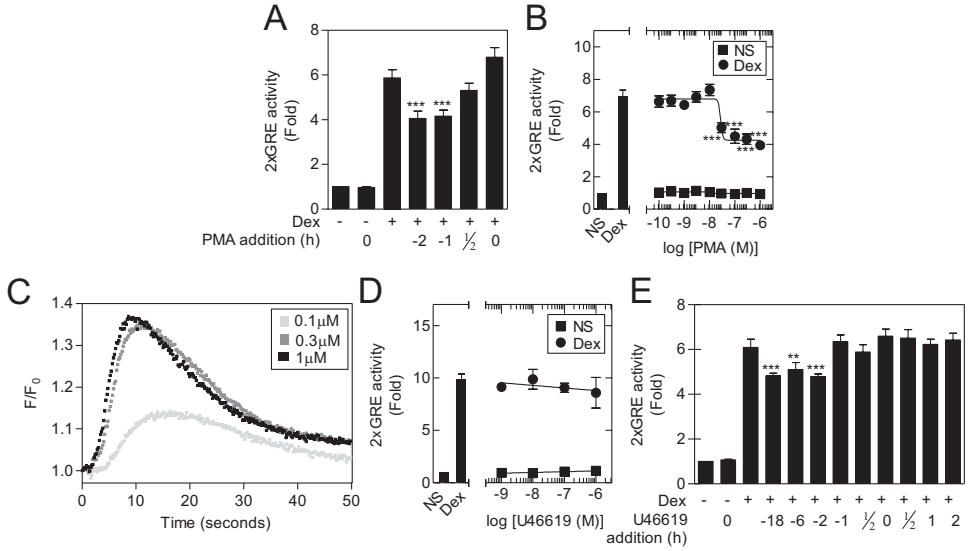

Fig. 4. Effects of PMA and the thromboxane analog U46619 on simple GRE-dependent transcription. A, BEAS-2B cells harboring a 2×GRE-dependent luciferase reporter were pretreated for up to 2 h, or not, with PMA (100 nM) before treatment with dexamethasone (1 μM) (Dex). Cells were harvested 6 h after Dex addition for luciferase assay. Data (n = 10), expressed as fold activation, are plotted as means ± S.E. B, BEAS-2B cells harboring a 2×GRE reporter were pretreated for 1 h, or not, with various concentrations of PMA before Dex (1 μM) addition. Cells were harvested after 6 h for luciferase assay. Data (n = 6), expressed as fold activation, are plotted as means ± S.E. C, BEAS-2B cells were loaded with Fluo-4. With excitation at 494 nm, fluorescence was measured at 516 nm for 1.6 s to determine baseline values (F_0). U46619 was injected to the indicated concentrations and fluorescence (F) measured for a further 48.4 s. Data (representative of three experiments), are expressed as F/F_0, which is a proxy for intracellular calcium concentration, $[Ca^{2+}]_i$. D, BEAS-2B 2×GRE reporter cells were treated, or not, with the indicated concentrations of U46619 for 1 h before treatment with Dex (1 μM). Cells were harvested after 6 h for luciferase assays. Data (n = 6), expressed as fold activation, are plotted as means ± S.E. E, BEAS-2B cells harboring a 2×GRE reporter were treated with U46619 (1 μM) for up to 18 h before and 2 h after the addition of Dex (1 μM). Cells were harvested 6 h after Dex addition for luciferase assay. Data (n = 6), expressed as fold activation, are plotted as means ± S.E. Statistical analyses, relative to the Dex control, were performed by ANOVA with a Dunnett's post-test. **, $P < 0.01$; ***, $P < 0.001$.

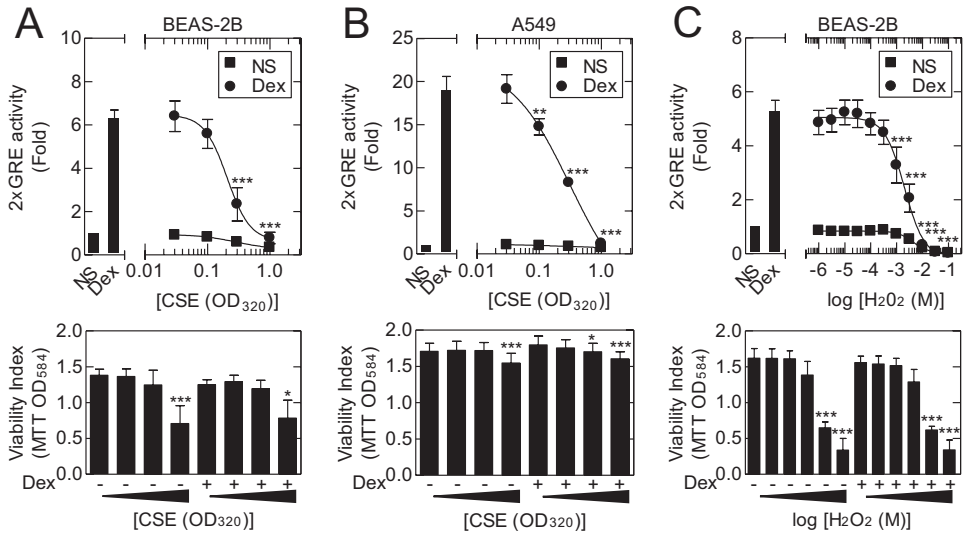

Fig. 5. Effects of CSE and H_2O_2 on 2×GRE-dependent transcription. A and B, BEAS-2B cells (A) or A549 cells (B), harboring a 2×GRE-dependent luciferase reporter, were pretreated, or not, with the indicated concentrations of CSE (expressed as OD_{320}) for 1 h before treatment, or not, with Dex (1 μM). Cells were harvested after 6 h for luciferase assay determination. Data (A, n = 10; B, n = 7), expressed as fold activation, are plotted as means ± S.E. Cell viability (bottom panels) was determined in parallel plates through incubation of cells with MTT (1 mg/ml) for 30 min before lysis with DMSO. Data (A, n = 5; B, n = 7), expressed as absorbance at 584 nm (OD_{584}), are plotted as means ± S.E. Concentrations of CSE used were 0.03, 0.1, 0.3, and 1 expressed as OD_{320}. C, BEAS-2B 2×GRE reporter cells were pretreated, or not, with the indicated concentrations of H_2O_2 for 1 h before treatment with dexamethasone (1 μM) (Dex). Cells were harvested after 6 h for luciferase assay determination. Data (n = 9), expressed as fold activation, are plotted as means ± S.E. Cell viability (bottom panel) was determined by MTT assay. Data (n = 5), expressed as OD_{584}, are plotted as means ± S.E. Concentrations of H_2O_2 ranged from 10^{-6} to 10^{-1} M. Statistical analyses, versus Dex (top panels) or untreated/Dex controls as appropriate (bottom panels), were performed by ANOVA with a Dunnett's post-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Effects of Proinflammatory Stimuli on the Expression of $p57^{KIP2}$ and GILZ. A549 cells were treated with various concentrations of dexamethasone in the presence or absence of IL-1β (1 ng/ml). Total RNA was harvested after 6 h, and the expression of $p57^{KIP2}$ and GILZ was examined by real-time PCR. As described previously (Kaur et al., 2008), both genes were induced highly by dexamethasone (Fig. 7A). Alone IL-1β had no significant effect on the expression of either gene. However, the presence of IL-1β dramatically reduced the $p57^{KIP2}$ expression that was induced by dexa-

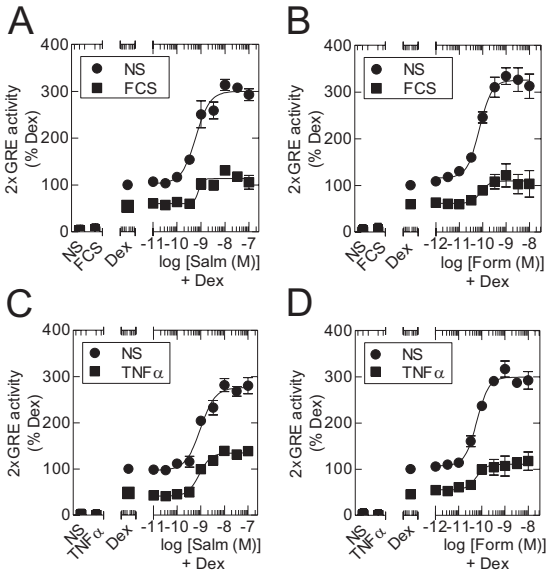

Fig. 6. Repression of 2×GRE-dependent transcription by FCS or TNFα is rescued functionally by LABAs. BEAS-2B cells harboring a 2×GRE-dependent luciferase reporter were pretreated, or not, with either 10% FCS (A and B) or TNFα (10 ng/ml) (C and D) for 1 h before addition, or not, of dexamethasone (1 μM) (Dex) and treatment, or not, with the indicated concentrations of salmeterol (A and C) or formoterol (B and D). Cells were harvested after 6 h for luciferase assay determination. Data (n = 5), expressed as a percentage of 1 μM Dex, are plotted as means ± S.E.

methasone. Like the simple GRE reporter, IL-1β did not affect sensitivity to dexamethasone (EC_{50} value without IL-1β = 1.45 ± 2.64 × 10^{-8} M; EC_{50} value with IL-1β = 2.17 ± 1.93 × 10^{-8} M), but the maximal response (efficacy) was attenuated significantly (Fig. 7A). In contrast, there was no obvious effect of IL-1β on the induction of GILZ mRNA by dexamethasone. To explore the generality of this result, the effect of TNFα was tested on the dexamethasone-induced mRNA expression of $p57^{KIP2}$ and GILZ in BEAS-2B cells (Fig. 7B). As in the A549 cells, mRNAs for both genes were induced highly by dexamethasone. However, whereas dexamethasone-induced $p57^{KIP2}$ again was repressed markedly by TNFα, there was no significant effect on GILZ expression. In the presence of formoterol, which had no effect alone, the ability of dexamethasone to induce $p57^{KIP2}$ mRNA was potentiated significantly (Fig. 7B). Likewise, in the presence of TNFα, formoterol restored the dexamethasone-induced level of $p57^{KIP2}$ mRNA to a level that was similar to that observed in the absence of TNFα (Fig. 7B). Thus, formoterol functionally restored this aspect of the response to glucocorticoids that was attenuated previously by TNFα. Despite this, it is salient to note that the induction of $p57^{KIP2}$ mRNA by dexamethasone plus formoterol nevertheless was reduced significantly by the presence of TNFα. GILZ expression was not altered significantly in the presence of formoterol. These findings were corroborated by Western blot analysis for both $p57^{KIP2}$ and GILZ proteins (Fig. 7C).

Discussion

Numerous cytokines including IL-1, IL-2, IL-4, TNFα, and interferon γ are described as reducing various aspects of GR activity (Kam et al., 1993; Sher et al., 1994; Pariante et al., 1999; Irusen et al., 2002; Tliba et al., 2008). For example, in peripheral blood mononuclear cells, IL-2 plus IL-4 reduces the affinity of glucocorticoid for GR (Kam et al., 1993; Irusen et al., 2002). Likewise, a combination of TNFα plus interferon γ reduces the response to glucocorticoids in human airway smooth muscle cells (Tliba et al., 2008). Similarly, IL-1α may reduce GR translocation and transcriptional activation in the mouse fibroblast line L929 (Pariante et al., 1999). With respect to IL-2, this effect may involve the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway (Tsitoura and Rothman, 2004). In peripheral blood mononuclear cells from steroid-resistant patients, c-fos (FBJ murine osteosarcoma viral oncogene homolog) expression is reported to be up-regulated, leading to increased AP-1 activity and reduced GR activity (Lane et al., 1998). More recently, many authors have focused on how these changes may relate to the ability of GR to repress the expression of inflammatory genes. Thus, the recruitment of HDAC2 to the promoters of inflammatory genes forms a key component of the transrepressive ability of GR (Barnes, 2006; Barnes and Adcock, 2009). Furthermore, reduced HDAC2 expression, possibly due to enhanced degradation (Adenuga et al., 2009), may explain the glucocorticoid resistance that is observed in chronic obstructive pulmonary disease (Ito et al., 2005). Equally, smoke inhalation in mice or cells treated with CSE show reduced HDAC2 activity, and this is consistent with reports of reduced repressive abilities of glucocorticoids in alveolar macrophages from chronic obstructive pulmonary disease patients or after treatment with CSE (Culpitt et al., 2003; Adenuga et al., 2009; Marwick et al., 2009).

However, the repression of inflammatory gene expression by glucocorticoids also involves the up-regulation of anti-inflammatory genes (Newton and Holden, 2007; Clark et al., 2008). Therefore, we examined a number of common stimuli, including proinflammatory cytokines, mitogens, such as FCS and PMA, as well as CSE and oxidative stress, on the ability of glucocorticoids to induce transcriptional responses. We found significant reductions in the levels of simple GRE-dependent transcription after pretreatment with IL-1β, TNFα, PMA, FCS, and CSE. These effects were observed in two models of lung epithelial cells, suggesting that the current data are generally applicable. Indeed, similar responses using GRE reporters also are reported in human airway smooth muscle cells and mouse fibroblasts (Pariante et al., 1999; Tliba et al., 2008). Furthermore, although a modest increase in the EC_{50} value to glucocorticoid was found after TNFα and FCS treatment in A549 cells, the biological significance of this is questionable. More importantly, we show that the reduced responses described with the simple GRE reporter also apply to the bona fide glucocorticoid-inducible gene $p57^{KIP2}$. Given biological properties, including the repression of cell proliferation and the attenuation of JNK signaling (Samuelsson et al., 1999; Chang et al., 2003), which are consistent with the effects of glucocorticoids, it is not unreasonable to predict that loss of glucocorticoid-inducible gene expression may affect functional responses. In certain patients, this may contribute to reduced responses to ICS therapy.

More importantly, our data predict that simply increasing the dose of glucocorticoid will not overcome this resistance, because the maximal inducibilities of the GRE reporter and of $p57^{KIP2}$ expression were repressed. This correlates with

TABLE 1

Effects of FCS on the activation of 2×GRE-dependent transcription and the enhancement by LABAs

BEAS-2B cells harboring a 2×GRE reporter were not treated (naive) or were treated with formoterol (10 nM) (Form) or salmeterol (100 nM) (Salm) in the presence of either no stimulation (NS) or dexamethasone (1 μM) (Dex). Cells were pretreated for 1 h, or not, with 10% FCS. After 6 h, cells were harvested for luciferase assay. Data are from Fig. 6 plus additional experiments and are expressed as fold activation ± S.E. relative to untreated. In addition, enhancement by LABAs was calculated relative to naive or dexamethasone treatment.

	NS		Dex			FCS		FCS + Dex		
	N	Activation	*N*	Activation	Enhancement by LABA	*N*	Activation	*N*	Activation	Enhancement by LABA
Naive	14	1.0	14	7.1 ± 0.3	1	14	1.2 ± 0.1	14	3.8 ± 0.3§§§	1
Form	4	1.0 ± 0.0	9	21.1 ± 1.4***	3.0 ± 0.2	4	0.9 ± 0.3	9	6.5 ± 1.1*,§§§	2.2 ± 0.5#
Salm	4	0.9 ± 0.0	9	21.5 ± 0.9***	3.0 ± 0.1	4	0.8 ± 0.3	9	7.0 ± 1.0**,§§§	2.0 ± 0.1###

ANOVA with a Dunnett's multiple comparison to compare the activation by Dex with activation by Dex plus Form or Salm (in both the absence and the presence of FCS): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Paired *t* test to examine the effects of FCS on the enhancement by Dex, Dex + Form, and Dex + Salm: §§§ $P < 0.001$. Paired *t* test (one-tailed) between the enhancement by LABA in the absence and presence of FCS: # $P < 0.05$; ### $P < 0.001$.

TABLE 2

Effects of TNFα on the activation of 2×GRE-dependent transcription and the enhancement by LABAs

BEAS-2B cells harboring a 2×GRE reporter were not treated (naive) or were treated with formoterol (10 nM) (Form) or salmeterol (100 nM) (Salm) in the presence of either no stimulation (NS) or dexamethasone (1 μM) (Dex). Cells were pretreated for 1 h, or not, with TNFα (10 ng/ml). After 6 h, cells were harvested for luciferase assay. Data are from Fig. 6 plus additional experiments and are expressed as fold activation ± S.E. relative to untreated. In addition, enhancement by LABAs was calculated relative to naive or dexamethasone treatment.

	NS		Dex			TNFα		TNFα + Dex		
	N	Activation	*N*	Activation	Enhancement by LABA	*N*	Activation	*N*	Activation	Enhancement by LABA
Naive	20	1.0	20	7.0 ± 0.4	1	20	1.1 ± 0.2	20	3.6 ± 0.2§§§	1
Form	10	1.0 ± 0.1	15	18.4 ± 1.5***	2.7 ± 0.1	10	0.9 ± 0.1	15	7.5 ± 0.6***,§§§	2.1 ± 0.1###
Salm	10	1.0 ± 0.1	15	18.7 ± 1.6***	2.8 ± 0.2	10	1.0 ± 0.1	15	9.3 ± 0.6***,§§§	2.6 ± 0.1 N.S.

N.S., not significant.

ANOVA with a Dunnett's multiple comparison to compare the activation by Dex with activation by Dex plus Form or Salm (in both the absence and the presence of TNFα): *** $P < 0.001$. Paired *t* test to examine the effects TNFα on the enhancement by Dex, Dex + Form, and Dex + Salm: §§§ $P < 0.001$. Paired *t* test (one-tailed) between the enhancement by LABA in the absence and presence of TNFα: ### $P < 0.001$.

the clinical situation, where increasing the concentration of the ICS is only of modest benefit. Thus, clinical guidelines (http://www.ginasthma.com) recommend the addition of a LABA to an ICS rather than simply increasing the dose of an ICS (Giembycz et al., 2008). Because LABAs can enhance GR-dependent gene transcription to levels that cannot be achieved by glucocorticoid alone (Kaur et al., 2008), we were prompted to explore the effects of LABAs in the context of induced reductions in steroid responsiveness. We now show that, whereas TNFα and FCS repress dexamethasone-dependent transcription, the clinically relevant LABAs formoterol or salmeterol restored GRE-dependent transcription back to the level that was achieved by dexamethasone alone (in the absence of an inflammatory stimulus). Although there was some evidence that the presence of TNFα or FCS also reduced the level of simple GRE enhancement produced by the LABAs, this still represents a significant improvement in glucocorticoid action. More importantly, the effect was confirmed at the level of $p57^{KIP2}$ expression and therefore offers considerable mechanistic insight as to how LABAs could, by increasing the expression of otherwise repressed glucocorticoid-induced genes, act to overcome glucocorticoid resistance in patients. Thus, concentration-dependent increases in glucocorticoid-dependent transcription that are produced by LABAs may contribute to both enhanced protection from severe exacerbations and steroid-sparing in patients whose ICS + LABA combination therapy is intensified, as needed, on top of regular maintenance therapy. Such advantages were shown using a budesonide/formoterol combination inhaler (Symbicort) for Maintenance and Reliever Therapy (Symbicort SMART), which was found to produce the greatest therapeutic benefit in patients with severe asthma and with a high risk of exacerbation (Peters, 2009). Notwithstanding such effects, the fact that expression of $p57^{KIP2}$, induced by dexamethasone plus formoterol, was significantly reduced by inflammatory stimuli also indicates that there may be considerable further ability to improve the therapeutic potential of ICS + LABA combination therapies.

Although the mechanisms underlying the repression of glucocorticoid-induced transcription were not addressed specifically in the current study, other investigators have variously indicated reduced binding affinity of ligand for GR, reduced expression and/or translocation of GR, as well as reduced ability to interact with AP-1, phosphorylation of GR, or the induction of GRβ, as potential mechanisms to account for resistance (Adcock et al., 1995ab; Webster et al., 2001; Irusen et al., 2002; Matthews et al., 2004). These possibilities require investigation in the context of the current findings. Despite this, the current study is important, because it provides a valid alternative to effects on transrepression in explaining steroid resistance. Thus, reduced expression of key glucocorticoid-induced effector genes may lead to clinically relevant attenuation of glucocorticoid efficacy. Likewise, the ability to enhance the expression of these same genes with a LABA, in the context of a glucocorticoid, may account for the enhanced clinical benefit of combining a LABA with a regular ICS. An important point to come from these data is that it should be possible to indentify inhibitors of signaling pathways that prevent the repression of GR-dependent gene expression by inflammatory stimuli such as TNFα or possibly cigarette smoke. The data reported in the current study should encourage the continued analysis of glucocorticoid-inducible genes as well as the pathways that may lead to reductions in their expression in the context of inflammation.

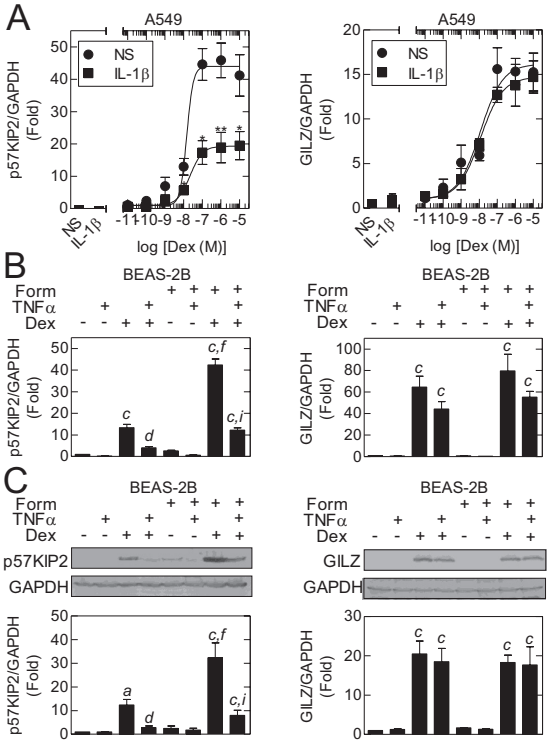

Fig. 7. Effects of IL-1β and TNFα on the expression of $p57^{KIP2}$ and GILZ. A, A549 cells were either not stimulated (NS) or treated with IL-1β in absence or presence of the indicated concentrations of dexamethasone (Dex). After 6 h, cells were harvested, and total RNA was extracted. After cDNA synthesis, real-time PCR was performed for the glucocorticoid-inducible genes $p57^{KIP2}$ and GILZ. Data ($n = 6$), expressed as fold, are plotted as means ± S.E. B, BEAS-2B cells harboring a 2×GRE-dependent luciferase reporter were treated, or not, with TNFα (10 ng/ml) for 1 h before treatment, or not, with Dex (1 μM) and/or formoterol (10 nM) (Form). Cells were harvested 6 h after the addition of Dex, and total RNA was extracted. After cDNA synthesis, real-time PCR was performed for the genes $p57^{KIP2}$ and GILZ. Data ($n = 4$), expressed as fold activation, are plotted as means ± S.E. C, BEAS-2B cells were pretreated, or not, with TNFα for 1 h before the addition of Dex (1 μM) and/or formoterol (10 nM). Cells were harvested for protein 6 h after the addition of Dex. Cell lysates were subjected to Western blot analysis for GILZ, $p57^{KIP2}$, and glyceraldehyde 3-phosphate dehydrogenase. Blots representative of three such experiments are shown. After densitometric analysis, data, expressed as fold induction, are plotted as means ± S.E. A, statistical analyses comparing the effect of IL-1β with control for each concentration of Dex was by paired t test. *, $P < 0.05$; **, $P < 0.01$. B and C, the effect of TNFα on Dex or Dex + Form induced $p57^{KIP2}$ was assessed by ANOVA with a Bonferroni's post-test. a, $P < 0.05$ relative to control; c, $P < 0.001$ compared with control; d, $P < 0.05$ compared with Dex; f, $P < 0.001$ relative to Dex; i, $P < 0.001$ relative to Dex + Form.

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Authorship Contributions

Participated in research design: Rider, Giembycz, and Newton.

Conducted experiments: Rider, King, Holden, and Newton.

Performed data analysis: Rider, King, Holden, and Newton.

Wrote or contributed to the writing of the manuscript: Rider, King, Holden, Giembycz, and Newton.

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Address correspondence to: Dr. Robert Newton, Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. E-mail: rnewton@ucalgary.ca

Supplemental data

Inflammatory stimuli inhibit glucocorticoid-dependent transactivation in human pulmonary epithelial cells: Rescue by long-acting β_2-adrenoceptor agonists

Christopher F. Rider, Elizabeth M. King, Neil S. Holden, Mark A. Giembycz and Robert Newton

Airways Inflammation Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada.

(C.F.R., E.M.K., N.S.H., M.A.G., R.N.)

Corresponding author: Dr. Robert Newton

Address: Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1

Tel: 001 403 210 3938 Fax: 001 403 210 7944 E-mail: rnewton@ucalgary.ca

SUPPLEMENTAL TABLE 1

Effect of inflammatory cytokines and fetal calf serum on the sensitivity of simple GRE-dependent transcription to glucocorticoid.

A. BEAS-2B 2×GRE cells: Effect of TNFα (10 ng/ml)

Treatment	*N*	EC_{50}	SE
Dexamethasone	6	4.42×10^{-8}	1.26×10^{-8}
Dexamethasone + TNFα	6	5.98×10^{-8}	1.54×10^{-8}
Budesonide	6	5.46×10^{-9}	0.54×10^{-9}
Budesonide + TNFα	6	7.38×10^{-9}	0.85×10^{-9}
Fluticasone propionate	6	1.08×10^{-8}	0.22×10^{-8}
Fluticasone propionate + TNFα	6	1.01×10^{-8}	0.15×10^{-8}

B. BEAS-2B 2×GRE cells: Effect of fetal calf serum (10%) (FCS)

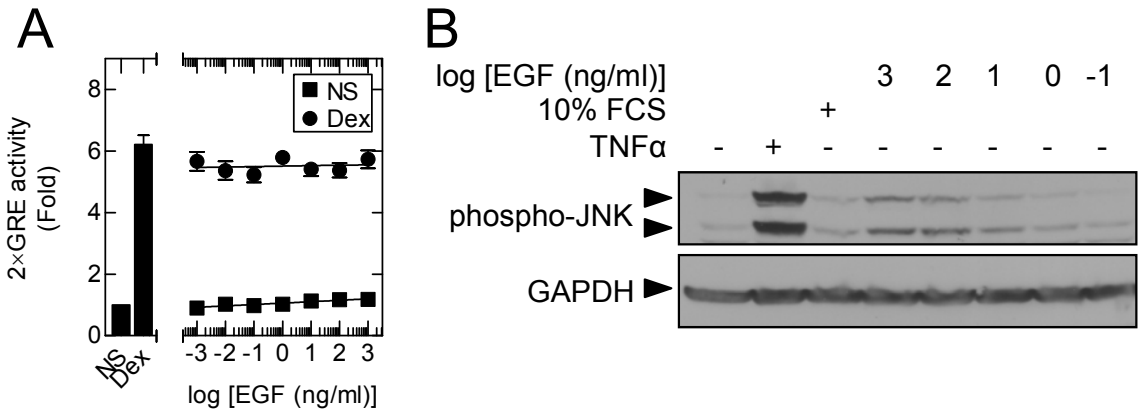
Treatment	*N*	EC_{50}	SE
Dexamethasone	4	2.98×10^{-8}	0.85×10^{-8}
Dexamethasone + FCS	4	2.61×10^{-8}	0.27×10^{-8}
Budesonide	6	6.25×10^{-9}	0.48×10^{-9}
Budesonide + FCS	6	1.24×10^{-8}	0.30×10^{-8}
Fluticasone propionate	6	7.89×10^{-9}	0.74×10^{-9}
Fluticasone propionate + FCS	6	9.05×10^{-9}	0.15×10^{-9}

C. A549 2×GRE cells: Effect of TNFα (10 ng/ml), IL-1β (1 ng/ml) and fetal calf serum (10%)

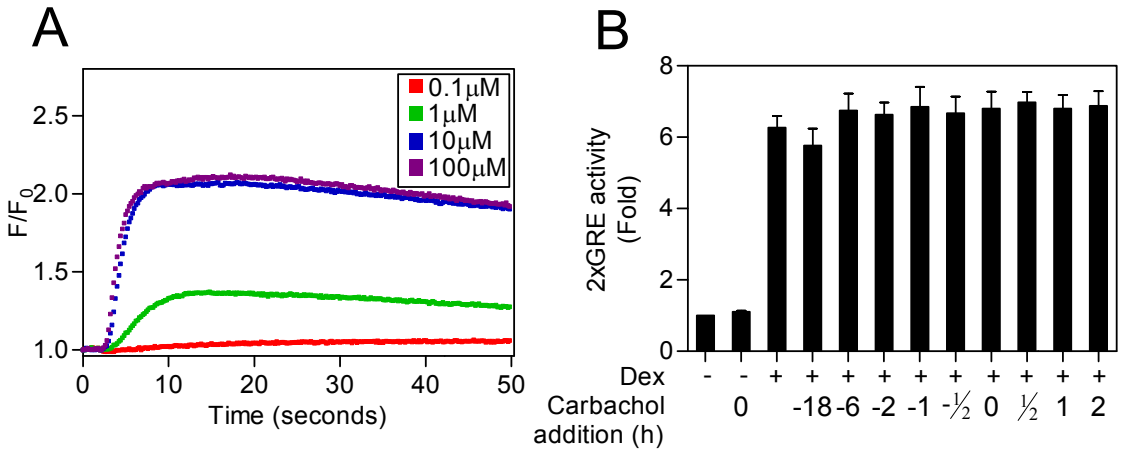
Treatment	*N*	EC_{50}	SE	
Dexamethasone	6	6.56×10^{-8}	0.71×10^{-8}	] *
Dexamethasone + TNFα	6	8.85×10^{-8}	0.99×10^{-8}	
Dexamethasone	6	7.23×10^{-8}	0.57×10^{-8}	
Dexamethasone + IL-1β	6	1.36×10^{-7}	0.52×10^{-7}	
Dexamethasone	8	5.67×10^{-8}	0.17×10^{-8}	] **
Dexamethasone + FCS	8	7.27×10^{-8}	0.54×10^{-8}	

Each data set for the curves depicted in Figure 3 was analyzed individually to derive individual EC_{50} values. Mean EC_{50} ± SE were calculated in respect of each glucocorticoid in the absence and presence of FCS (10%), TNFα (10 ng/ml) or IL-1β (1 ng/ml). All experimental conditions are as described in Fig. 3. Significance between means was tested by paired t test. * $P < 0.05$, ** $P < 0.01$.

SUPPLEMENTAL FIGURES



Supplemental Fig. 1. Effect of EGF on 2×GRE-dependent transcription. A, BEAS-2B cells harbouring a 2×GRE-dependent luciferase reporter were pre-treated, or not, with various concentrations of epidermal growth factor (EGF) for 1 h prior to treatment with dexamethasone (1 μM) or not (naive). The cells were harvested after 6 h for luciferase assay determination. Data (n = 8), expressed as fold activation, are plotted as means ± SE. B, BEAS-2B cells were treated with TNFα (10 ng/ml), 10% FCS or various concentrations of EGF. After 15 mins, cells were harvested for western blot analysis of phospho-JNK and GAPDH. Blots representative of 4 such experiments are shown.



Supplemental Fig. 2. Effect of EGF on 2×GRE-dependent transcription. A, BEAS-2B cells were loaded with Fluo-4. Using excitation at 494 nm, fluorescence was measured at 516 nm for 1.6 seconds to determine baseline values (F_0). Carbachol was injected to the indicated concentrations and fluorescence (F) measured for a further 48.4 seconds. Data (representative of 4 experiments), are expressed as F/F_0, which is a proxy for intracellular calcium concentration, $[Ca^{2+}]_i$. B, BEAS-2B cells harbouring a 2×GRE-dependent luciferase reporter were pre-treated, or not, with various concentrations of carbachol for 1 h prior to treatment with dexamethasone (1 μM). The cells were harvested after 6 h for luciferase assay determination. Data (n = 4-6), expressed as fold activation, are plotted as means ± SE.