Inhaled Budesonide Enhances Effector Gene Expression in Humans: A Randomized Controlled Trial and Comparison with *In Vitro* Analysis

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Rationale & Hypothesis

Glucocorticoids act on the glucocorticoid receptor (GR/NR3C1) and, given as inhaled corticosteroids (ICSs), reduce inflammation in asthma by reducing inflammatory gene expression. Mechanisms by which corticosteroids reduce inflammatory gene expression may include the ability of GR to directly bind to key transcription factors, such as NF-κB, and thereby reduce inflammatory gene transcription. However there is an increasing body of data suggesting that corticosteroids may induce the expression of multiple anti-inflammatory or anti-asthma genes to exert their effects. While this statement is supported *in vitro*, there is a deficit in respect of relevant clinical information *in vivo* in humans. The current study was therefore designed to test the *hypothesis* that the ICS, budesonide, will induce the expression of multiple anti-inflammatory genes *in vivo* in humans.

Methods & Clinical Protocol

- Twelve healthy non-smoking, non-atopic male volunteers with normal lung function were enrolled into this prospective double-blind, placebo-controlled, randomised, two-period cross-over study (**Fig. 1**). Participant eligibility criteria are listed in **Table 1**.
- A single dose of inhaled placebo or budesonide (1600 µg) was administered by Turbuhaler®. Bronchoscopy was performed 5-6 h later, when endobronchial brushings and biopsies were obtained and samples processed for histology and gene expression analysis.
- A549 cells, primary human bronchial epithelial cells (HBE) (from, brushings), airways smooth muscle (ASM), human bronchial fibroblasts (fibroblasts) and human umbilical vein endothelial (endothelial) cells were cultured according to standard procedures.
- Total RNA was extracted using RNeasy kits (Qiagen). RNA was reverse transcribed to cDNA, and SYBR Green PCR was carried out for the indicated genes and GAPDH.
- CEL files from Affymetrix PrimeView microarrays were analysed with Partek Genomic Suite (v6.6).
- Serum budesonide concentrations were measured by LC-MS/MS.

Table 1: Eligibility criteria for inclusion of healthy volunteers

- Non-smoker males aged 18-50 years, not on ICS or other corticosteroid treatments
- Negative skin prick test to common aero-allergens
- Normal lung function ($FEV_1/FVC \geq 0.7$, $FEV_1 \geq 80\%$)
- Normal airway responsiveness (PC_{20} methacholine > 16 mg/ml)
- No exposure to corticosteroids in the preceding 3 months
- No participation in any other clinical study in the preceding 4 weeks
- No associated morbidity where bronchoscopy was contraindicated

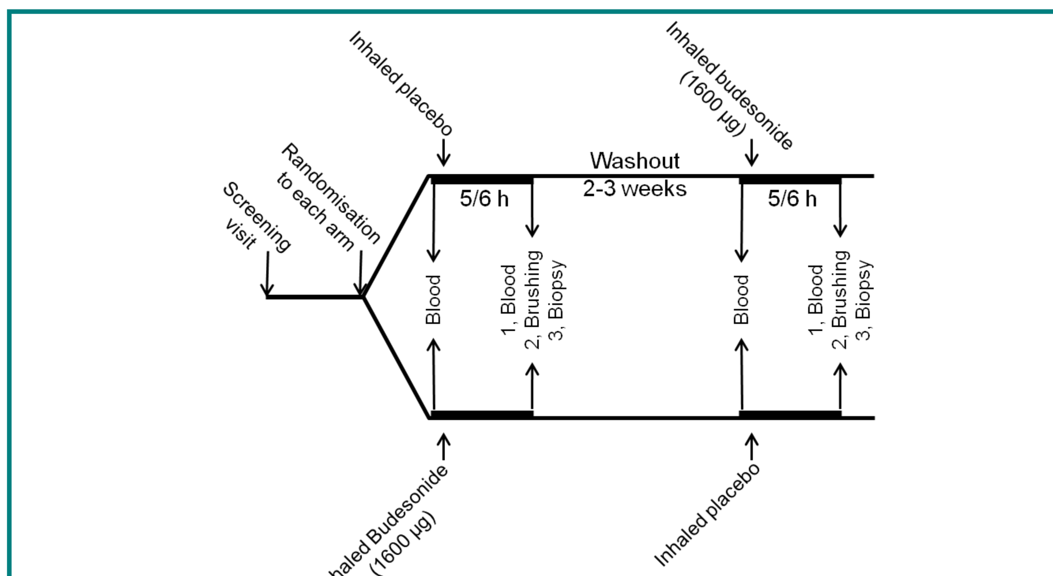


Fig. 1. Study design. Following screening, study participants were randomized to receive either inhaled placebo followed by inhaled budesonide, or inhaled budesonide followed by inhaled placebo. Treatment interventions were separated by a 2-3 week washout period. Randomization and treatment allocation was by research pharmacy personnel, not otherwise associated with the study.

Results

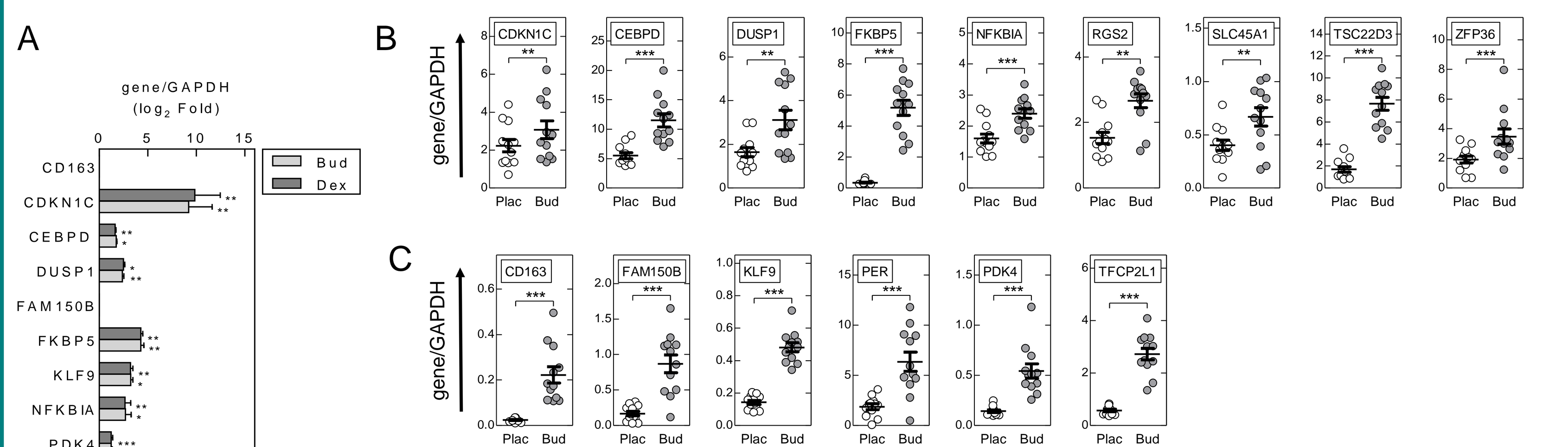


Fig. 2. RT-PCR analysis of A549 cells and bronchial biopsies. A, A549 cells were either not stimulated or treated with budesonide (0.3 µM) (Bud) or dexamethasone (1 µM) (Dex). Real-time PCR was performed. Data, n = 8 are plotted as $\log_2$ fold as means ±SE. Significance was tested by paired ANOVA with a Dunn's post test. **B,** Biopsy samples obtained after placebo and budesonide (1600 µg) inhalation were subjected to real-time PCR. Data, from 12 individuals, are plotted as means ±SE. Significance was assessed by Wilcoxon signed rank test). C, Following microarray analysis, expression of additional genes was examined by real-time PCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

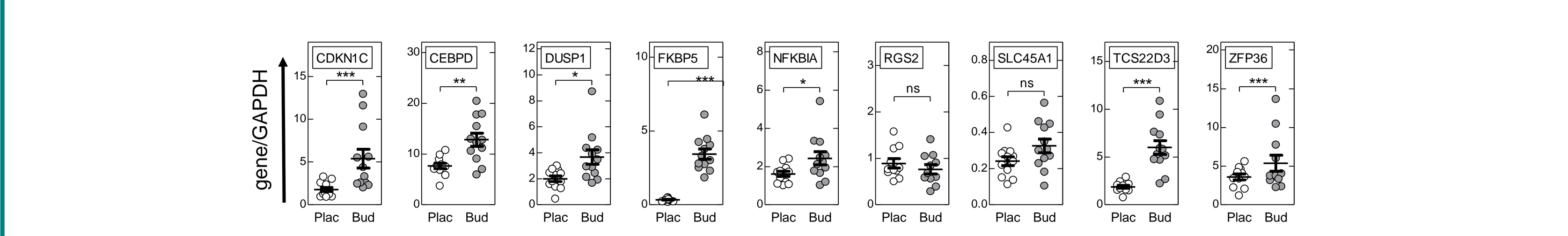


Fig. 3. RT-PCR analysis of bronchial brushings. Bronchial brushings collected after placebo and budesonide (1600 µg) inhalation were subjected to real-time PCR. Data, from 12 individuals, are plotted as means ±SE. Significance was assessed by Wilcoxon signed rank test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

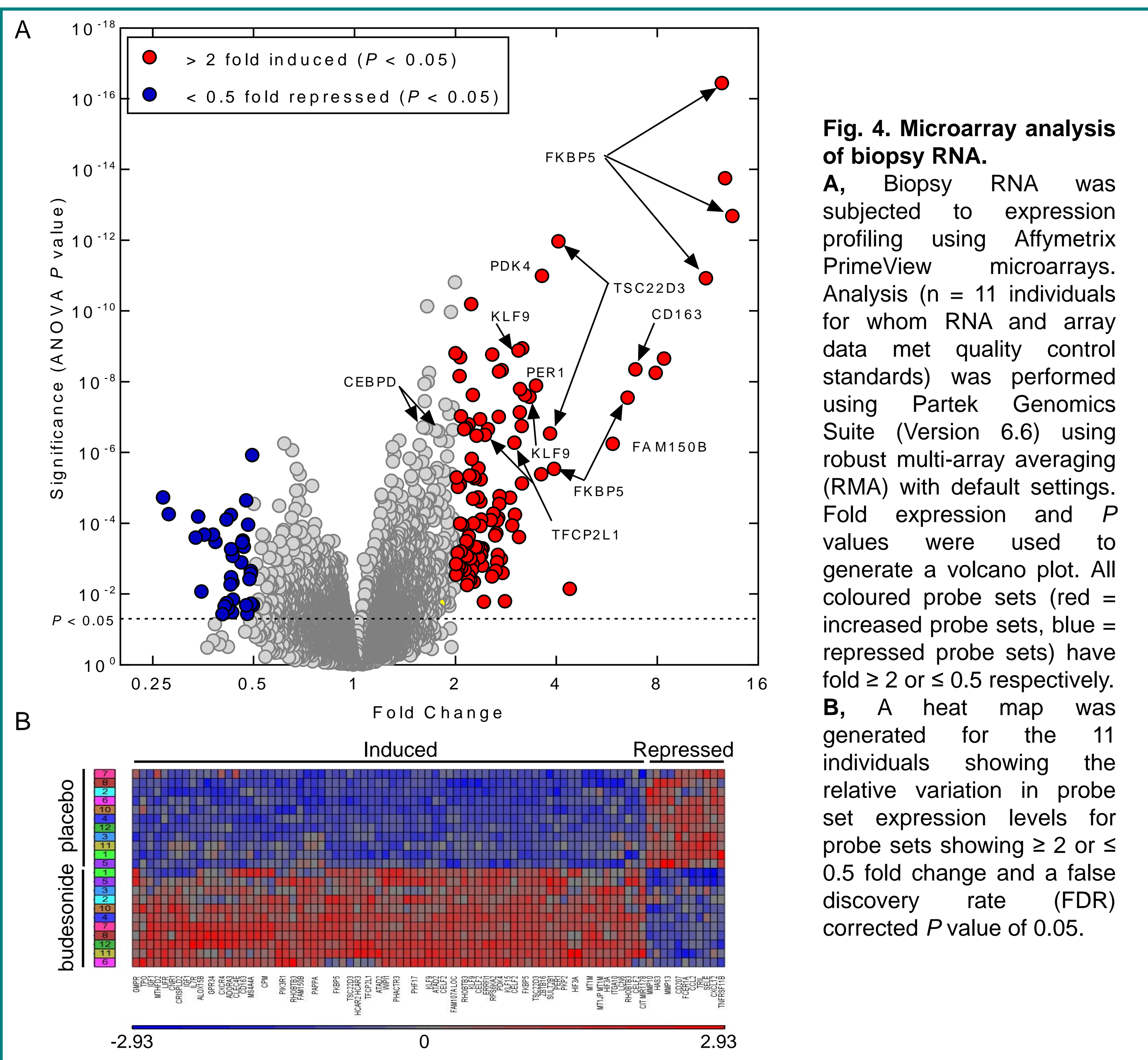


Fig. 4. Microarray analysis of biopsy RNA. A, Biopsy RNA was subjected to expression profiling using Affymetrix PrimeView microarrays. Analysis (n = 11 individuals for whom RNA and array data met quality control standards) was performed using Partek Genomics Suite (Version 6.6) using robust multi-array averaging (RMA) with default settings. Fold expression and *P* values were used to generate a volcano plot. All coloured probe sets (red = increased probe sets, blue = repressed probe sets) have fold ≥ 2 or ≤ 0.5 respectively. **B,** A heat map was generated for the 11 individuals showing the relative variation in probe set expression levels for probe sets showing ≥ 2 or ≤ 0.5 fold change and a false discovery rate (FDR) corrected *P* value of 0.05.

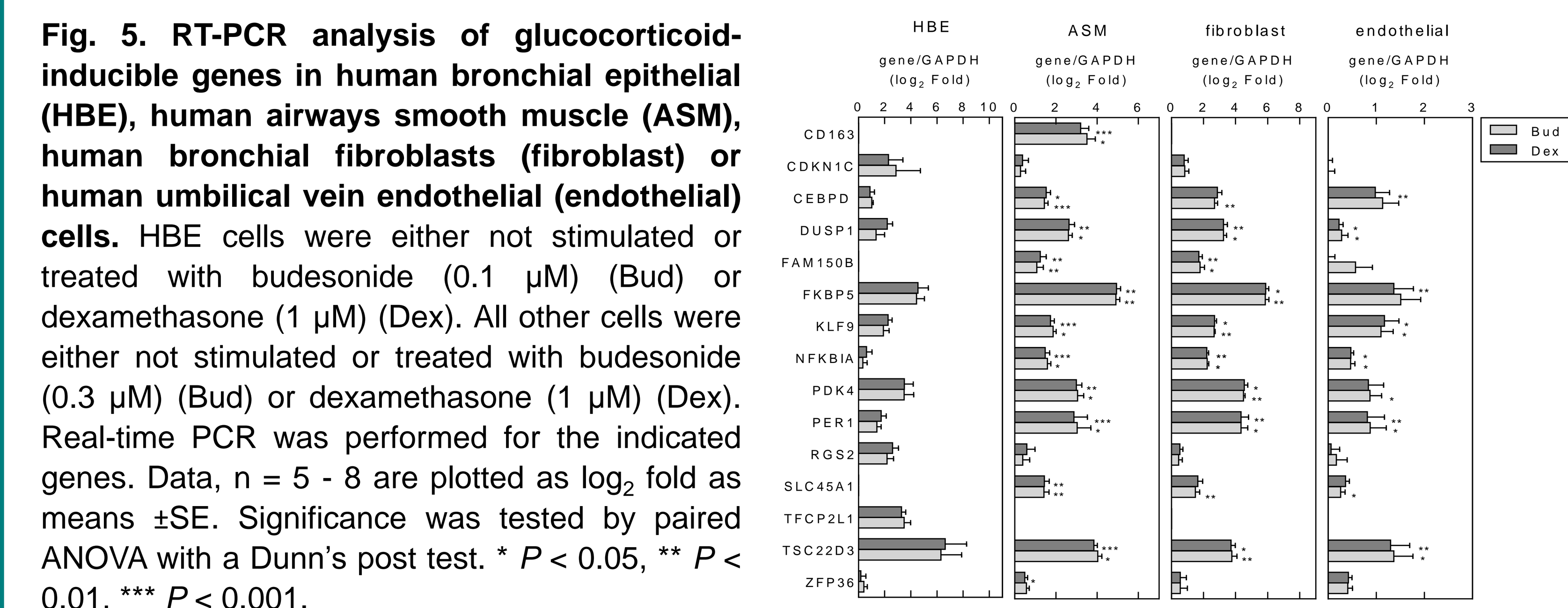


Fig. 5. RT-PCR analysis of glucocorticoid-inducible genes in human bronchial epithelial (HBE), human airways smooth muscle (ASM), human bronchial fibroblasts (fibroblast) or human umbilical vein endothelial (endothelial) cells. HBE cells were either not stimulated or treated with budesonide (0.1 µM) (Bud) or dexamethasone (1 µM) (Dex). All other cells were either not stimulated or treated with budesonide (0.3 µM) (Bud) or dexamethasone (1 µM) (Dex). Real-time PCR was performed for the indicated genes. Data, n = 5 - 8 are plotted as $\log_2$ fold as means ±SE. Significance was tested by paired ANOVA with a Dunn's post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

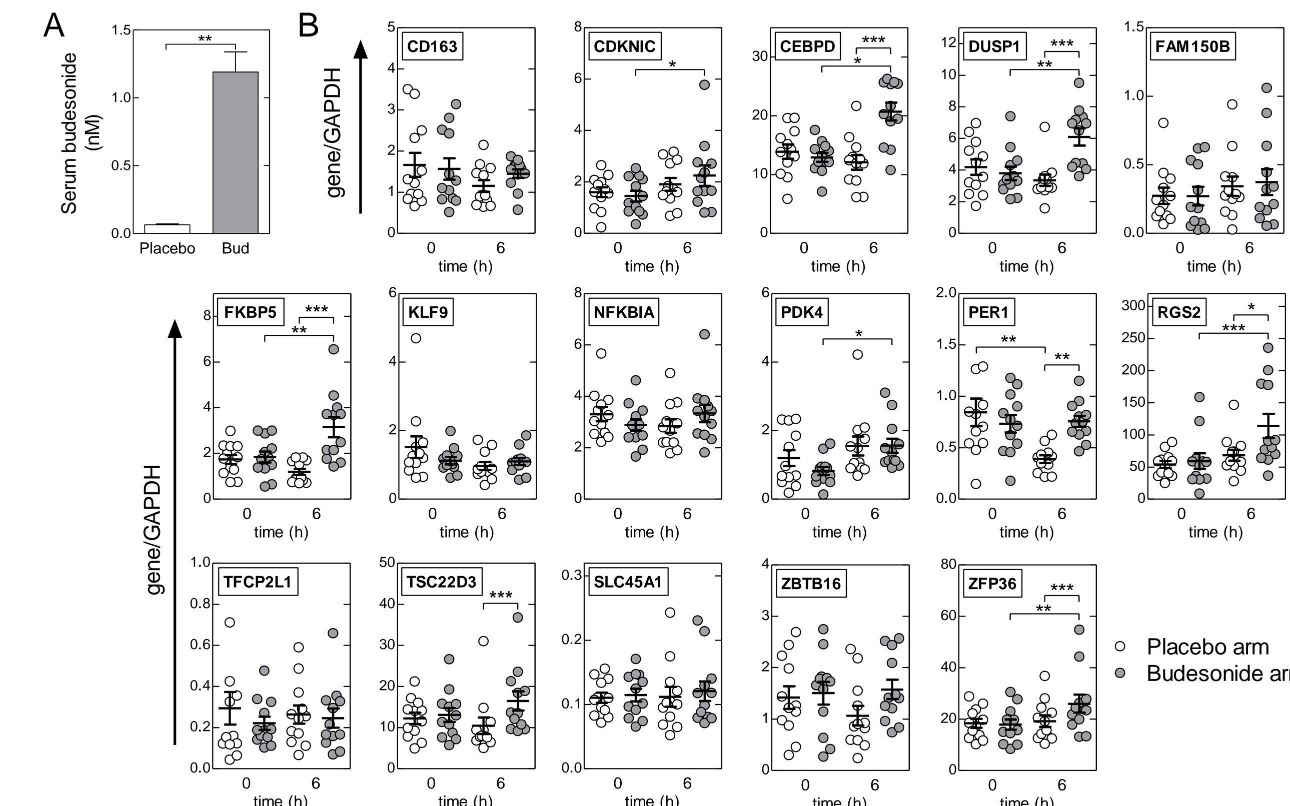
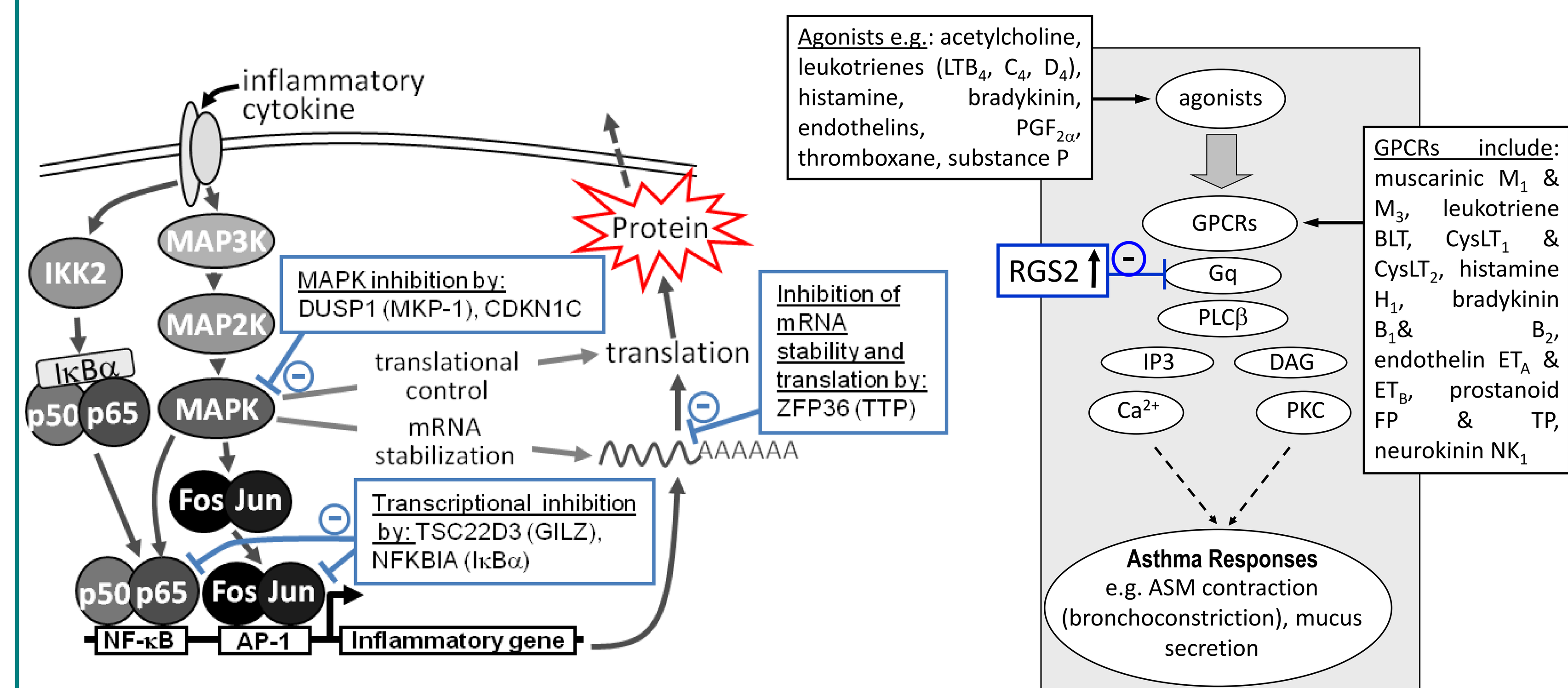


Fig. 6. Serum budesonide and corticosteroid-induced gene expression in the blood. A, Blood was collected 5-6 h after placebo or budesonide (1600 µg) inhalation. Serum was analysed by LC-MS/MS to determine budesonide concentration. The assay limit was 0.05 nM and samples with no detectable budesonide were assigned a value of 0.05 nM. Data (n = 12 individuals) are plotted as means ± SE. Significance was tested by Wilcoxon signed rank test. **B,** RNA was prepared from whole blood and PCR performed for the indicated genes. Data, (n = 12 individuals) are plotted as means ±SE. Significance was assessed by ANOVA with a Dunn's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Conclusions and Clinical Relevance



- Previous analyses identified FKBP5 (FKBP51), TSC22D3 (GILZ), DUSP1 (MKP-1), ZFP36 (TTP), CDKN1C (p57KIP2), CEBPD (C/EBPδ), NFKBIA (IκBα), RGS2 (RGS2) and SLC45A1 (DNB5) as being induced by dexamethasone in A549 cells *in vitro*. We now reconfirm these effects in A549 cells and show that such effects also occur *in vivo* in humans following inhalation of budesonide.
- The mRNA expression of these genes, and many others, was increased in bronchial biopsies following inhalation of a single, clinically relevant dose (1600 µg) of budesonide. Similar effects were observed in bronchial brushings and other relevant cell types present in the airways.
- These data confirm that inhaled budesonide significantly up-regulates the expression of multiple glucocorticoid-inducible genes. This, together with our earlier finding that TCS22D3 was up-regulated in mild asthmatic subjects taking a medium dose of inhaled budesonide over a 10 day period (*Kelly et al., 2012 Brit. J. Pharmacol. 165, 1737*), strongly suggests that the ability to induce the expression of such gene products could contribute to the therapeutic effects of ICS.
- The therapeutic potential of up-regulating many of these genes is clear. Thus: i) RGS2 protects airway smooth muscle from contractile responses (*Holden et al. 2011 Proc. Natl. Acad. Sci. USA 108, 19713*); ii) DUSP1 dephosphorylates and inactivates MAPKs; iii) NFKBIA and TSC22D3 inhibit the inflammatory transcription factor, NF-κB; iv) ZFP36 promotes mRNA degradation of multiple inflammatory mRNAs; and v) CDKN1C is anti-proliferative and may attenuate JNK activity. The above schematics illustrate biological effects of up-regulating the expression of these gene products.

Conclusion. The current data strongly support the contention that enhanced expression (i.e. *transactivation*) of multiple corticosteroid-induced genes represents an clinically important mode of anti-inflammatory / anti-asthma action following inhalation of ICSs, as shown here for budesonide.

Acknowledgments

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