Additive anti-inflammatory effect of formoterol and budesonide on human lung fibroblasts

F M Spoelstra, D S Postma, H Hovenga, J A Noordhoek, H F Kauffman

Background: It has been shown that treatment with a long acting β2 agonist in addition to a glucocorticoid is beneficial in the treatment of asthma. In asthma inflammatory cells, particularly eosinophils, migrate into the pulmonary tissue and airway lumen by means of adhesion molecules expressed on resident tissue cells—that is, fibroblasts—and become activated by cytokines and adhesion interactions. A study was undertaken to determine whether an interaction exists between the long acting β2 agonist formoterol and the glucocorticoid budesonide on inhibition of adhesion molecule expression, as well as chemokine/chemokine production by human lung fibroblasts.

Methods: Lung fibroblasts were preincubated with therapeutically relevant drug concentrations of 10–4 M to 10–6 M. Cells were stimulated with interleukin (IL)-1β [1 or 10 U/ml] for 8 hours and supernatants were collected for measurement of GM-CSF and IL-8 concentrations. The cells were fixed and subjected to a cell surface ELISA technique to measure the expression of ICAM-1 and VCAM-1.

Results: Formoterol exerted an additive effect on the inhibition of IL-1β stimulated ICAM-1 and VCAM-1 upregulation and GM-CSF production by budesonide in concentrations of 10–6 M and above (p<0.05). IL-8 production was not influenced by formoterol.

Conclusion: Formoterol exerts an additive effect on the anti-inflammatory properties of budesonide. In vitro data support the finding that the combination of budesonide and formoterol in asthma treatment strengthens the beneficial effect of either drug alone.
Europe Ltd, Badhoevedorp, The Netherlands) and used for experiments. Fibroblast characterisation was performed with antibodies against vimentin, cytokeratin, desmin, smooth muscle actin, and fibronectin using fluorescence microscopy. Fibroblast purity was more than 98%, the only contaminating cells being smooth muscle cells. Two other lung fibroblast strains derived from tissue of two different non-asthmatic individuals were also used and comparable results were achieved.

**Drugs**

Budesonide was obtained from a Pulmicort Turbuhaler (Astra Pharmaceutica BV, Zoetermeer, The Netherlands) and dissolved in 96% ethanol in a concentration of 10⁻⁶ M. Subsequently, solutions of 10⁻⁹ to 10⁻¹⁰ M budesonide (considered therapeutically relevant concentrations) were prepared in Ham’s complete medium. Formoterol fumarate dihydrate (Astra Draco AB, Lund, Sweden) was dissolved in DMSO in a concentration of 10⁻⁶ M, followed by stimulation for 8 hours with 1 U/ml IL-1β (to mimic a chronic inflammatory environment) (Boehringer Mannheim, Mannheim, Germany) in the presence of budesonide, formoterol, or a combination of the two drugs (total volume 100 µl). Ethanol and DMSO were used as vehicle controls for budesonide and formoterol, respectively. Ham’s complete medium was used as baseline control.

**Inhibition of ICAM-1 and VCAM-1 expression**

Confluent fibroblast layers in 96-well plates were preincubated for 45 minutes with different concentrations of budesonide and/or formoterol (10⁻⁶–10⁻⁹ M) followed by stimulation for 8 hours (providing optimal expression) with 1 U/ml IL-1β to mimic a chronic inflammatory environment (Boehringer Mannheim, Mannheim, Germany) in the presence of budesonide alone, formoterol alone, or a combination of the two drugs (total volume 100 µl). Ethanol and DMSO were used as vehicle controls for budesonide and formoterol 10⁻⁹ M, respectively. Ham’s complete medium was used as baseline control.

**Cytokine production**

Confluent fibroblast layers in 24-well plates were preincubated for 45 minutes with different concentrations of budesonide and/or formoterol, followed by stimulation for 8 hours with 10 U/ml IL-1β (Boehringer Mannheim, Mannheim, Germany) in the presence of budesonide, formoterol, or a combination of the drugs (total volume 1 ml). Ham’s complete medium was used as a control. Cell-free media were stored at –80°C for later measurement of cytokine concentrations using GM-CSF (R&D Systems) and IL-8 (CLB, Amsterdam, The Netherlands).

**Table 1** Effect of IL-1β stimulation on adhesion molecule expression and cytokine production of human lung fibroblasts

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<tr>
<th>Activation parameter</th>
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<th>IL-1β stimulated</th>
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<tr>
<td>ICAM-1 (OD₄₉₀nm)</td>
<td>0.264 (0.168–0.404)</td>
<td>2.461 (2.301–2.604)*</td>
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<tr>
<td>VCAM-1 (OD₄₉₀nm)</td>
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Optical density (OD) and concentrations are represented as median (interquartile range) values. *p<0.05 compared with unstimulated human lung fibroblasts.

In additional experiments, 1 hour before incubation of the combined drugs the cells were preincubated either with budesonide alone or formoterol alone in order to test possible divergent effects resulting from a difference in the sequence of addition. Subsequently, fibroblasts were washed twice with 200 µl cold PBS supplemented with 0.01% CaCl₂ and fixed for 10 minutes in 96% ethanol at 4°C. Fibroblasts were dried on air for 30 minutes and stored at 4°C for maximally 14 days until determination of adhesion molecule expression.

Determination of ICAM-1 and VCAM-1 expression was performed using a modified CSE assay according to Piela and coworkers as described recently. Briefly, non-specific binding sites on fibroblast layers were blocked with 1% bovine serum albumin (BSA) and subsequently incubated with anti-ICAM-1, anti-VCAM-1 (R&D Systems, Abingdon, UK) or IgG₁, isotype control (CLB, Amsterdam, The Netherlands) for 120 minutes at room temperature. Fibroblast layers were washed and incubated with HRP conjugated rabbit anti-mouse antibody (DAKO A/S, Glostrup, Denmark) for 30 minutes. After the second washing step, substrate solution containing o-phenylene diamine dihydrochloride (Sigma Chemical Co, St Louis, MO, USA) was added and colour development was stopped after 30 minutes by 3 M H₂SO₄. Absorbance at 490 nm was measured using a microplate reader.

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**Figure 1** Effects of budesonide (open bars), formoterol (dark shaded bars) or both (light shaded bars) in concentrations of 10⁻⁶–10⁻⁹ M on (A) ICAM-1 and (B) VCAM-1 upregulation of human lung fibroblasts induced by IL-1β (1 U/ml). Results are presented as median percentages of inhibition with interquartile range (n=6). *p=vehicle control of 10⁻⁹ M budesonide, formoterol, or both. *p<0.05 compared with expression after IL-1β stimulation, #p<0.05 compared with inhibition of budesonide and formoterol alone (additive effect).
enzyme linked immunosorbent assay (ELISA) kit, according to the manufacturer’s instructions.

Data analysis
ICAM-1 and VCAM-1 expression was calculated as the mean values of optical density (OD, 490 nm) from quadruplicate determinations within one experiment after subtracting OD values of IgG, isotype control. Coefficients of variation did not exceed 10%; outlying OD values within quadruplicates were only omitted when they exceeded 2SD values of the mean. To evaluate statistical differences the mean OD of the quadruplicate determinations representing ICAM-1 and VCAM-1 expression and GM-CSF and IL-8 concentrations in pg/ml were tested using the non-parametric Wilcoxon signed rank test for related samples and Friedman’s test. Six separate experiments were performed. Differences were considered significant at p < 0.05. Percentages of inhibition are presented as median values with interquartile ranges. IC\textsubscript{50} values were calculated from the individual concentration response curves by linear regression and are presented as median values with interquartile ranges.

**RESULTS**

**Inhibition of ICAM-1 and VCAM-1 upregulation on lung fibroblasts**

IL-1\(\beta\) upregulated ICAM-1 and VCAM-1 expression significantly on human lung fibroblasts (table 1). Budesonide and formoterol both inhibited IL-1\(\beta\) induced ICAM-1 (fig 1A) and VCAM-1 (fig 1B) upregulation in a dose dependent manner, being significant at concentrations of 10\textsuperscript{–9} M and 10\textsuperscript{–8} M. Inhibition by the combination of budesonide and formoterol was significantly larger than with either of them used alone in all assessed concentrations. Furthermore, inhibition of ICAM-1 and VCAM-1 upregulation occurred at a concentration of 10\textsuperscript{–9} M (16 (9–36)% and 47 (8–56)% respectively). Formoterol had mainly an additive effect on the inhibition of IL-1\(\beta\) induced ICAM-1 and VCAM-1 upregulation by budesonide on lung fibroblasts. Vehicle controls for 10\textsuperscript{–9} M did not significantly influence ICAM-1 and VCAM-1 upregulation, except for significantly enhanced VCAM-1 upregulation by ethanol (budesonide vehicle control, 124 (95–176)%; fig 1B). IC\textsubscript{50} values for budesonide, formoterol, and the combination of the two drugs are presented in table 2.

We also assessed the effect of the combination of budesonide and formoterol when one of the two was preincubated for 1 hour before the combined drugs to determine any possible divergent effects when the sequence of addition of formoterol and budesonide was changed. No significant differences were found between the additional preincubation of the separate drugs and the simultaneous incubation of budesonide and formoterol (data not shown).

**Inhibition of GM-CSF and IL-8 production by lung fibroblasts**

Human lung fibroblasts produced large quantities of the cytokine GM-CSF and the chemokine IL-8 after IL-1\(\beta\) stimulation (table 1). Budesonide and formoterol significantly inhibited IL-1\(\beta\) induced GM-CSF production in a dose dependent manner at all concentrations tested (fig 2A). The combination of budesonide and formoterol significantly inhibited GM-CSF production by 66 (47–71)% at 10\textsuperscript{–9} M, but this was not significantly different from the inhibition by budesonide alone (58 (42–63)%). At concentrations of 10\textsuperscript{–9} M and 10\textsuperscript{–8} M the inhibition by the combination of drugs was significantly larger than the inhibition induced by similar concentrations of the separate drugs, but not larger than the sum of inhibition. These effects were therefore defined as additive. Vehicle

![Figure 2](http://www.thoraxjnl.com)

**Figure 2**. Effects of budesonide (open bars), formoterol (dark shaded bars) or both (light shaded bars) in concentrations of 10\textsuperscript{–10}–10\textsuperscript{–4} M on (A) GM-CSF and (B) IL-8 production of human lung fibroblasts induced by IL-1\(\beta\) (10 U/ml). Results are presented as median percentages of inhibition with interquartile range (n=6). * = vehicle control of 10\textsuperscript{–9} M budesonide, formoterol, or both. # = p<0.05 compared with expression after IL-1\(\beta\) stimulation; ## = p<0.05 compared with inhibition of budesonide and formoterol alone (additive effect).

**Table 2**. Median (interquartile range) IC\textsubscript{50} values of budesonide, formoterol, and the combination of the two drugs on inhibition of GM-CSF production and ICAM-1 and VCAM-1 upregulation by human lung fibroblasts after IL-1\(\beta\) stimulation

<table>
<thead>
<tr>
<th>Activation parameter</th>
<th>IC\textsubscript{50} budesonide</th>
<th>IC\textsubscript{50} formoterol</th>
<th>IC\textsubscript{50} combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>2.2 [0.7–2.9] \times 10\textsuperscript{4}</td>
<td>2.6 [1.6–4.3] \times 10\textsuperscript{4}</td>
<td>7.7 [7.0–18.2] \times 10\textsuperscript{-10}</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1.4 [1.1–6.8] \times 10\textsuperscript{4}</td>
<td>1.0 [0.5–2.4] \times 10\textsuperscript{4}</td>
<td>2.1 [1.0–3.0] \times 10\textsuperscript{-10}</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.9 [0.8–1.0] \times 10\textsuperscript{10}</td>
<td>7.9 [7.1–9.0] \times 10\textsuperscript{-10}</td>
<td>0.8 [0.7–0.9] \times 10\textsuperscript{-10}</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.5 [0.8–2.6] \times 10\textsuperscript{10}</td>
<td>–</td>
<td>1.8 [1.0–4.4] \times 10\textsuperscript{-10}</td>
</tr>
</tbody>
</table>
controls for budesonide and formoterol, but not the combina-
tion, significantly enhanced GM-CSF production by 120 (105–
129) and 120 (109–122), respectively. IC50 values for
budesonide, formoterol, and the combination of the two drugs
are presented in table 2. IL-8 production after IL-1β stimula-
tion was significantly inhibited by budesonide in a dose dependent
manner but, in contrast to the effect on GM-CSF, there was no additive effect
of formoterol at the concentrations assessed (fig 2B). Moreover, formoterol alone did not significantly inhibit IL-8
production. Vehicle controls did not influence IL-8 production
significantly.

Additional preincubation of either budesonide or formot-
erol separately 1 hour before the combination of the drugs was
added showed a significantly stronger inhibitory effect on
GM-CSF production than standard preincubation (not shown).
This stronger inhibitory effect did not apply to IL-8
production.

DISCUSSION

This study shows that budesonide inhibits both GM-CSF and
IL-8 production of human lung fibroblasts after IL-1β
stimulation. Formoterol exerts an additive effect on the inhi-
bition of IL-1β induced GM-CSF production, but not on inhi-
bition of IL-8 production by budesonide. Formoterol also
exerts mainly an additive effect on the inhibition of IL-1β
induced ICAM-1 and VCAM-1 upregulation by budesonide.

Our observations suggest that the combination of budeso-
nide and formoterol as therapeutic treatment may have an
increased inhibitory effect on chronic inflammation in the air-
ways of asthmatic individuals compared with the separate use
of these drugs. Upregulation of adhesion molecules on
resident pulmonary cells is diminished, which may lead to
reduced infiltration of inflammatory cells. Production of
GM-CSF is also diminished, which may lead to decreased
activation and chemotaxis of eosinophils in the pulmonary
tissue. Prevention of migration and activation of inflammatory
cells probably results in better control of chronic and acute
inflammation and less bronchoconstriction and hypererespon-
siveness.

Our in vitro data are in accordance with data from clinical
studies evaluating the effect of formoterol in addition to gluo-
corticoids. It has been shown that formoterol has an
additive effect on inhaled glucocorticoids in reducing symp-
toms and the number of exacerbations and improving morn-
ing peak expiratory flow (PEF) and forced expiratory volume
in 1 second (FEV1).2 Salmeterol and salbutamol alone did not
inhibit IL-8 release from human airway smooth muscle cells
but they enhanced the inhibition induced by dexamethasone
and fluticasone.3 Salbutamol alone had an inhibitory effect on
ectokin production by human airway smooth muscle cells,
and this effect was stronger when salbutamol was combined
with dexamethasone or fluticasone.4 Results of an in vivo
study in which reversion or prevention of formoterol induced
β adrenoceptor tolerance by systemic glucocorticoids was
found also support the beneficial effects of combining
glucocorticoids and β agonists.5 In contrast, there are also
reports in which glucocorticoids do not prevent the develop-
ment of tolerance induced by β agonists.6

There are several reports of antagonistic actions of β
agonists and glucocorticoids at the cellular level. Kankaan-
ranta et al7 reported that, in contrast to glucocorticoids, β
agonists delayed eosinophil apoptosis. The addition of
salmeterol (long term exposure) to dexamethasone resulted
in an antagonistic effect on the inhibition of superoxide
production by eosinophils.8 In the same in vitro study an
antagonistic effect of albuterol on dexamethasone induced
eosinophil apoptosis was also found. On the other hand,
albuterol did not antagonise the inhibition of GM-CSF and
TNFα production of monocytes by budesonide9 and the long
acting β2 agonist salmeterol had an additive effect on the inhibi-
tion of GM-CSF production in blood mononuclear cells by
dexamethasone.10 Discrepancies in the abovementioned stud-
ies may be the result of differences in cell specificity, in the
measured inflammatory variables, in the incubation time of
the drug, or in the nature of the stimulus used.

Glucocorticoids act via AP-1 and NFκB by binding of GC-GR
to these transcription factors.2 Through this mechanism they
probably influence gene transcription of adhesion
molecules11 and cytokines.12 Formoterol stimulates β
adrenoceptors which causes activation of the adenyl cyclases
ystem and a rise in intracellular cAMP levels, leading to PKA
activation. PKA, in turn, is able to activate PKA responsive
element binding protein (CREB). CREB can interact with
transcription factors such as AP-1 and NFκB, thus interfering
with gene transcription.13

There are different possible interactions between gluco-
corticoids and β agonists ranging from synergistic or additional
effects to antagonistic effects.14 The additive effects reported
here probably result from the fact that both CREB and the
GC-GR complex bind the transcription factors that are neces-
sary for induction of gene transcription of ICAM-1, VCAM-1,
and GM-CSF. An alternative possibility is that glucocorticoids
increase transcription of the β adrenoceptor in lung tissue15
and prevent desensitisation of this receptor after long term
exposure to β agonists.16

In contrast to GM-CSF production, IL-8 production is not
inhibited by formoterol in the same experimental setting.
However, IL-8 release from human smooth muscle cells is
inhibited by salmeterol, but only when steroids are given concomitantly.17

We found similar additive effects when the sequence of for-
moterol and budesonide addition was varied. Only with
GM-CSF production was the inhibition stronger when
budesonide or formoterol were added before the combination
of these drugs than without preincubation of the separate
drugs. This can be explained by the longer total time of prein-
cubation in the first two cases, and longer incubation times
would probably remove this difference. The fact that this was
not seen with ICAM-1 and VCAM-1 expression or IL-8
production may be because of the high sensitivity of GM-CSF
production to the inhibitory effects of budesonide and
formoterol. In contrast to our findings, Korn et al18 found an
inhibitory effect of a β agonist on the activity of a glucocorti-
coid. The β agonist terbutaline inhibited the downregulation
of α and β GR mRNA by budesonide in epithelial cells, but this
effect was abrogated when budesonide was applied 4 hours
before terbutaline. The authors concluded that, in order to
avoid deleterious interference of β agonists with the activity
of glucocorticoids, they should be administered in vivo a few
hours after the glucocorticoid. However, a clinical study found
no negative interaction when budesonide and terbutaline
were used in combination.19 Our results are in agreement with
the in vivo data.

In conclusion, formoterol had a mainly additive effect on
the inhibition of ICAM-1 and VCAM-1 upregulation and
GM-CSF production by budesonide in human lung fibroblasts.
Inhibition of IL-8 production by budesonide was not affected
by formoterol. Our in vitro data indicate that there is no rea-
son, at the level of human lung fibroblast activation, why
patients should not use these drugs simultaneously. As estab-
lished in a clinical study,2 the beneficial effects may partially
have been achieved through the additive inhibition of cellular
infiltration resulting from diminished adhesion molecule
expression and GM-CSF production of lung fibroblasts.

ACKNOWLEDGEMENTS

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References


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