Interactions between theophylline and salbutamol on cytokine release in human monocytes

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Running Title Page

Running Title: Usefull Interactions between theophylline and salbutamol

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Abbreviations: TNF-α, tumor necrosis factor-alpha; IL, interleukin; LPS, lipopolysaccharide; PCR, polymerase chain reaction; db-cAMP, N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt; PDE, phosphodiesterase; HDAC, histone deacetylase; HAT, histone acetyltransferase; NF-κB, nuclear factor-kappa B; PI3K, phosphoinositol-3–kinase: CRE, cAMP response element; CREB, cAMP response element binding protein; PKA, protein kinase A; COPD, chronic obstructive airway disease.

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Abstract

The combination of β₂-agonists with inhaled steroids has become the standard treatment for mild to moderate asthma. Theophylline has also been combined successfully with inhaled steroids. However, the possible interaction between theophylline and β₂-agonists, with regards to their anti-inflammatory effects, has not been clarified. The aim of this study was to investigate the in vitro interaction between theophylline and salbutamol on cytokine generation from human monocytes and to compare this with a similar interaction between dexamethasone and salbutamol. Purified monocytes from normal donors were pre-treated with the drugs (alone or in combination) and stimulated with lipopolysaccharide (LPS) for 24h. Released tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as well as their corresponding mRNA expressions, were determined and analyzed. Salbutamol (≥ 0.1µM) significantly inhibited the release of TNF-α, but also significantly enhanced that of IL-6. In contrast, theophylline (50µM), as well as dexamethasone (0.1µM) strongly inhibited the generation of both cytokines. Interestingly, when used in combination, the effects of theophylline and salbutamol were additive in inhibiting TNF-α release but theophylline blocked the IL-6-enhancing effect of salbutamol. A similar effect was seen when dexamethasone was combined with salbutamol. These results show that β₂-agonists have opposing effects on the generation of TNF-α and IL-6, but that when combined with clinically relevant concentrations of theophylline, the latter, like dexamethasone, was capable of augmenting the anti-inflammatory effects of the β₂-agonists while at the same time preventing their pro-inflammatory effect. Thus, theophylline may have potentially useful steroid-sparing effect.
Introduction

Beta-2-adrenoceptor agonists (β2-agonists) and to a lesser extent theophylline are widely-used bronchodilators employed in the treatment of bronchial asthma and chronic obstructive pulmonary diseases (COPD) (Barnes, 2006). Classically, their bronchodilatory effect is believed to be mediated by the elevation of intracellular cyclic adenosine monophosphate (cAMP) levels in bronchial smooth muscles. However, while theophylline accomplishes this, at least in part, by competitive inhibition of the phosphodiesterases (PDEs) – the enzymes that hydrolyze cAMP, β2-agonists do so by activating the adenylyl cyclase enzymes that catalyze the synthesis of cAMP. Unlike the short-acting β2-agonists (eg, salbutamol), which rely mainly on their bronchodilator effect for their therapeutic success, theophylline has been shown to possess additional significant anti-inflammatory effects (Sullivan et al., 1991; Lim et al., 2001; Barnes, 2003).

Pro-inflammatory cells such as T-cells, eosinophils, neutrophils, alveolar macrophages and monocytes, are known to play important roles in the initiation and propagation of the allergic lung inflammation that is characteristic of asthma (Gosset et al., 1991; Hamid et al., 2003; Peters-Golden, 2004). It is now believed that the anti-inflammatory effect of theophylline is due to its ability to inhibit the responses of these pro-inflammatory cells (Lim et al., 2001; Barnes, 2003). For example, it has been shown that therapeutic concentration of theophylline (55 – 110 µM) can significantly inhibit the expression and release of TNF-α, IL-8, IL-5 and IL-13 from human blood monocytes/alveolar macrophages *in vitro* (Spatafora et al., 1994; Kimura et al., 2003; Yao et al., 2005). In vivo, the drug has been shown to significantly reduce the number of eosinophils and T-cells present in the bronchoalveolar lavage fluid of asthmatics (Kidney et al., 1995; Lim et al., 2001).

The molecular mechanism of the anti-inflammatory effect of theophylline is incompletely understood, but it appears to be independent of PDE inhibition since the effect can be seen at concentrations of the drug that have practically no PDE-inhibitory effect (Lim et al., 2001; Barnes,
Other proposed mechanisms include adenosine receptor antagonism (Holgate et al., 1984; Yasui et al., 2000), inhibition of nuclear factor kappa B (NF-κB) (Umeda et al., 2002), inhibition of phosphoinositide-3-kinase (PI3K) (Foukas et al., 2002) or increased apoptosis (Yasui et al., 2000) among others. In addition, recent findings suggest that such anti-inflammatory effect can be accounted for by the ability of theophylline to activate the nuclear histone deacetylase (HDAC) – an enzyme that is involved in the switching off of the transcription of many cytokines genes in activated pro-inflammatory cells (Ito et al., 2002).

A number of in vitro studies have shown that β2-agonists may also have moderate, but statistically significant, inhibitory effects on the release of cytokines from activated pro-inflammatory cells (Seldon et al., 1995; 1998). However, unlike theophylline, β2-agonists and other agents that raise intracellular cAMP tend to enhance, rather than inhibit, the release of certain pro-inflammatory cytokines such as IL-6 and IL-8 (Kavelaars et al., 1997; Shames et al., 2001). This conflicting scenario of inhibiting the release of some inflammatory cytokines while enhancing others, may be one of the reasons why significant anti-inflammatory effect is rarely seen in asthmatics treated with salbutamol and other short-acting β2-agonists.

The combination of β2-agonists with inhaled steroids has become the standard treatment for mild to moderate asthma, since they seem to act in a complementary, and sometimes synergistic, manner (Barnes, 2006). Theophylline has also been combined with inhaled steroids or anti-leukotriene drugs with significantly better clinical outcomes (Ukena et al., 1997; Allayee et al., 2007). In contrast, the possible interaction between theophylline and the short-acting β2-agonists, especially with respect to their effect on the release of inflammatory cytokines, has not been clarified, although a synergistic effect has been reported for the combination of procaterol and theophylline on eosinophil degranulation (Fujisawa et al., 2002).

We have hypothesized that when used together, theophylline might be able to prevent the pro-inflammatory action of salbutamol, and hence spare the anti-inflammatory effect of the latter. The
purpose of this study was, firstly, to determine the effect of therapeutic concentrations of
theophylline and salbutamol, as well as their combination, on the release of TNF-α and IL-6 from
stimulated human blood monocytes, and secondly, to compare such effects with those of
dexamethasone and its combination with salbutamol.
**Methods**

*Isolation and culture of human monocytes*

Fresh blood samples from healthy donors were obtained from the Kuwait Central Blood Bank, after donor informed consent, and under a protocol approved by the Institutional Ethics Committee. Mononuclear cells were first isolated from heparinized blood by the gradient centrifugation method. Highly purified monocytes were then isolated immunomagnetically using the Monocyte Negative Isolation kit (Dynal, Oslo, Norway). The purity of the monocytes (CD14+), determined by flow cytometry was >98% and viability (by trypan blue exclusion method) was >97%. Monocytes were washed twice and re-suspended in endotoxin-free RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Sigma-Aldrich, St Louis, MO, USA). They were then plated at a concentration of 5x10^5 cells/ml in a 48-well plate, pre-incubated with the drugs - salbutamol, procaterol, theophylline, dexamethasone, dibutyryl cAMP (db-cAMP), forskolin, PGE₂ (all from Sigma-Aldrich) or drug combinations for 30 min before being stimulated with 250 ng/ml (or as otherwise stated) LPS (Sigma-Aldrich). In all cases, the drug remained present during the 24h of subsequent culture. In some drug interaction experiments, increasing concentrations of both theophylline and salbutamol were used either alone or in combination. After 24 h of culture in a humidified incubator at 37°C and 5% CO₂, the supernatants were recovered by centrifugation and stored at -40°C pending cytokine determination.

*Assay of Cytokines*

Culture supernatants were assayed for TNF-α and IL-6 by the enzyme immunoassay (EIA) method using assay kits supplied by R&D Systems (Minneapolis, MN, USA) and following the manufacturer’s instructions.
Determination of cytokine mRNA expression

The method employed was based on our previously published protocol (Abu-Ghefreh et al., 2009). Briefly, purified monocytes were adjusted to a concentration of $10^6$ viable cells/ml and 2 ml aliquots added to each well of a 6-well plate ($2 \times 10^6$ cells/well). Cells were pre-incubated with the various drugs/drug combinations or medium for 30 min before being stimulated with LPS (250 ng/ml) for 2 h (previously determined to be optimal). Total cellular RNA was then extracted from the adherent monocytes using Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to the manufacturer’s instructions. The extracted RNA (1 µg) was reverse-transcribed using the High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and then amplified by the semi-quantitative RT-PCR reactions. The sequence of the oligonucleotide primers were as follows: TNF-α (5’-GTGACAAGCCTGTAGCCCA-3’ and 5’-AAAGTAGACCTGCCCAGAC-3’), IL-6 (5’-ATGAACCTCCTTCTCCACAAGCGC-3’ and 5’-GAAGAGCCCTCAGGCTGACTG-3’) and β-actin (5’-CAGCGGAACCGCTCATTGCCAATGG-3’ and 5’-TCACCCACACTGTGCACCATCTACGA-3’). Thermo cycling was carried out according to the following profile: 95ºC for 5 min before the first cycle, 94ºC for 30 sec, annealing temp (61ºC for TNF-α, 65ºC for IL-6 and 64ºC for β-actin) for 30 sec and 72ºC for 30 sec repeated for 30 cycles and followed by a final extension at 72ºC for 5 min.

The amplified products were then electrophoresed through a 1.5 % agarose gel at 100 volts and the gel stained with ethidium bromide. The density of each band was quantified in a densitometer, using Alpha Imager Gel Documentation System (Alpha Innotech, San Leandro, CA, USA). The relative values of each sample were calculated by reference to the band density of the housekeeping gene (β-actin). The amplification product sizes for TNF-α, IL-6 and β-actin were 428 bp, 628 bp and 294 bp, respectively, as expected.
Analysis

All results were expressed as the mean ± S.E.M. for n number of independent experiments. Drug concentrations producing 50% of maximal inhibition of response (IC\textsubscript{50} values) or enhancement of response (EC\textsubscript{50} values), were calculated from the concentration-effect curves by non-linear regression analysis using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, U.S.A). Differences between mean values were analyzed either by One-sample t-test or by Analysis of variance (ANOVA) followed by Dunnett’s test, as appropriate. Differences were considered statistically significant when p < 0.05. Drug interaction was analyzed by the isobologram method (Tallarida 2001).
Results

Effect of β2-agonists on LPS-induced release of TNF-α and IL-6

As shown in figure 1A, pre-treatment of human monocytes with β2-agonists - salbutamol and procaterol, resulted in a significant and concentration-dependent inhibition of LPS-induced TNF-α release over 24h. The mean (95% confidence interval) of the concentrations achieving 50% of maximal inhibition (IC_{50} values) were 31.6 (17.3 – 52.2) nM and 57.8 (26.5 – 87.9) nM, for salbutamol and procaterol, respectively. However, maximal inhibitions were relatively modest, approximately 46% and 60%, for salbutamol and procaterol, respectively. In contrast, both drugs significantly enhanced the concurrently released IL-6 in the same concentration range at which they suppressed TNF-α release (Fig. 1B). The mean enhancements of 140 – 160%, obtained at 1 µM of both drugs, were statistically significant, p<0.05, n=6. In these experiments, the amounts of the cytokines produced over 24 h by the control (un-stimulated) cells were in the ranges of 0.02 - 0.06 ng/10^6 cells and 0.5 – 0.8 ng/10^6 cells for TNF-α and IL-6, respectively. The corresponding values for LPS (250 ng/ml)-stimulated, but un-treated cells were 0.3 – 2.5 ng/10^6 cells and 3.2 – 10.5 ng/10^6 cells, respectively.

Since these drugs are known to act by increasing intracellular cAMP (Bailly et al., 1990), their effects were compared with those of other agents that raise intracellular cAMP, namely PGE₂ (which, like β2-agonists, is a G_s-coupled receptor agonist), forskolin (a direct activator of adenylyl cyclase) and db-cAMP (a cell-permeant cAMP analogue). As shown in figure 2(A&B), all three drugs, to varying degrees, significantly suppressed TNF-α release while enhancing IL-6 production, thus suggesting that both actions are cAMP-dependent.

In contrast to the effects seen with agents that raise cAMP, theophylline produced a strong and concentration-dependent inhibition of the release of both TNF-α and IL-6 (fig. 3). The inhibitions were statistically significant at concentrations ≥ 30 µM, and at 100 µM, the release of both cytokines was completely abolished. In the concentration range 30 – 50 µM, which is marginally
below the typical bronchodilatory plasma concentration of the drug (55 -110 μM) (Barnes and Pauwels, 1994), theophylline achieved 45-60% inhibition of both responses.

Interaction of theophylline with β2-agonists on the release of TNF-α and IL-6

In view of the finding that β2-agonists enhance, while theophylline inhibits, IL-6 secretion in LPS-stimulated monocytes, it was of interest to determine the effect of the two drugs used in combination. As shown in figure 4, the combination of salbutamol (1 μM) and theophylline (50 μM) resulted in a more pronounced inhibitory effect on TNF-α release, (fig. 4A). On the IL-6 release, theophylline still maintained its inhibitory effect, while at the same time completely preventing the enhancing effect of salbutamol (fig. 4B).

To determine if this interesting ability of theophylline to prevent the IL-6-enhancing effect of salbutamol was peculiar to β2-agonists or common to all drugs that raise intracellular cAMP, the effects of the combination of 50 μM theophylline with 0.1 μM PGE2 or 50 μM db-cAMP were studied. As shown in figure 5 (A&B), the interaction of theophylline with these two agents resulted in the abolition of the ability of these drugs to enhance the release of IL-6, essentially in the same manner as for salbutamol. Thus, it appears that theophylline has a unique ability to block the pathway by which intracellular cAMP (irrespective of the manner of its generation) mediates the enhancement of IL-6 generation.

To determine if the differential effects on the release of the two cytokines is influenced by the degree of stimulation, we examined the effects of salbutamol (1 μM) and theophylline (50 μM) on cells stimulated with two extra LPS concentrations - low (50 ng/ml) and high (1000 ng/ml). As shown in figure 6 (A&B), the differential effect of salbutamol on the release of TNF-α and IL-6, as well as its interaction with theophylline, were similar at the two levels of stimulation and were comparable with the results obtained when cells were stimulated with the intermediate LPS concentration of 250 ng/ml (fig. 4). However, for IL-6, it appeared that the enhancement of the
release by salbutamol was weaker at the low, compared with the high, stimulation levels. However, the interaction with theophylline was similar at all levels of stimulation.

Further pharmacological analysis of the nature of the interaction between theophylline and salbutamol (additive or synergistic) using the isobologram method (Tallarida 2001), showed that the interaction was essentially additive for TNF-α inhibition (data not shown). Data for IL-6 were not amenable to this analysis, but were clearly antagonistic.

Interaction of steroids with β2-agonists on the release of TNF-α and IL-6

Following the observation that therapeutic concentration of theophylline could prevent the IL-6 enhancing effect of β2-agonists on human monocytes, it was of interest to determine if steroids, which are the mainstay of the anti-inflammatory therapy of asthma, exhibited a similar interaction with β2-agonists. As shown in figure 7A, dexamethasone inhibited LPS-induced release of TNF-α with a high potency (IC₅₀ = 50 nM) and efficacy (maximum inhibition) of 100% at 1 µM. In the same concentration range, the drug also inhibited IL-6 release (fig. 7B). When added together with salbutamol, the inhibitory effects of the two drugs on TNF-α release were essentially additive (fig. 7C), while the IL-6-enhancing effect of salbutamol was lost (fig 7D). Thus, dexamethasone appears to behave in a very similar manner as theophylline when combined with a β2 agonist.

To explore the mechanism by which theophylline acts, as well as interacts with β2 agonists, the effect of the drug, either alone or together with salbutamol, on the expression of mRNA for TNF-α and IL-6 was studied. As shown in figure 8 (A&B), pre-treatment of the cells with salbutamol (1 µM) or theophylline (50 µM) alone resulted in a small but significant reduction in LPS-induced up-regulation of the mRNA for TNF-α. When used in combination, the effects of the two drugs were essentially additive (lane 5). On the other hand, salbutamol increased the expression of mRNA for IL-6 (though this did not reach statistical significance), whereas theophylline significantly
suppressed the expression (fig. 8C&D). When used together, the result was a significant inhibition of IL-6 mRNA expression, p<0.05. As expected, dexamethasone (0.1 µM) strongly inhibited the expression of mRNA for both TNF-α and IL-6, whereas, db-cAMP (50 µM) strongly inhibited TNF-α mRNA expression but significantly enhanced that for IL-6. Thus, these effects on mRNA expression closely reflect those on cytokine protein secretion.
Discussion

The possibility that theophylline, by virtue of its anti-inflammatory effect, could also be combined with the β2-agonists to achieve a result similar to that seen when steroids are combined with β2-agonists, has hardly been addressed. In this study, we have investigated the *in vitro* anti-inflammatory effect of salbutamol (as a representative of the short-acting β2-agonists) and theophylline, when used alone and in combination, and compared the results with those obtained with a similar combination between salbutamol and dexamethasone. The results obtained showed, firstly, that salbutamol (and also procaterol), significantly inhibited the release of TNF-α from LPS-activated monocytes but also significantly enhanced the concomitant release of IL-6. These opposing actions of the β2-agonists on the release of pro-inflammatory mediators, which were largely independent of the degree of cytokine induction, appeared to be dependent on the generation of intracellular cAMP since the same effects were reproduced by other agents that elevate or mimic intracellular cAMP – PGE₂, forskolin and db-cAMP. Similar results have been reported for other cytokines such as IL-1 and IL-8 by other workers (Bailly et al., 1990; Kavelaars et al., 1997; Shames et al., 2001).

In contrast to the β2-agonists, theophylline exhibited a powerful inhibitory effect on the generation of both TNF-α and IL-6. Statistically significant effects were seen even below the generally accepted bronchodilatory plasma concentration range of the drug (55–110 µM or 10-20 mg/L) (Barnes and Pauwels, 1994; Ohta et al., 2004). Recent reports have shown that low dose theophylline, resulting in plasma concentrations lower than 50 µM, have significant in vivo anti-inflammatory effects in treated asthmatics (Barnes and Pauwels, 1994; Oliver et al., 2001; Ohta et al., 2004). Our present results are consistent with such clinical observations since at 30 - 50 µM, theophylline consistently achieved significant inhibition of the release of both TNF-α and IL-6 - both cytokines being important mediators in the pathophysiology of asthma (Gosset et al., 1991; Broide et al., 1992; Barnes et al., 1998; Thomas, 2001).
The combination of clinically-relevant concentration of theophylline (50 µM), with salbutamol (0.1 – 1.0 µM) produced an interesting result. While the inhibitory effects of the two drugs on TNF-α release were additive, theophylline completely abolished the IL-6-elevating effect of salbutamol while retaining its own inhibitory effect. A strikingly similar result was seen when salbutamol was combined with dexamethasone. Fujisawa and co-workers (2002) have previously reported a synergistic inhibition of human eosinophil degranulation by a combination of theophylline and procaterol, but no such studies have been done on monocyte cytokine release.

The effects of the drugs, when used alone or in combination were further verified at the molecular level by evaluating the mRNA expression for the two cytokines. Although salbutamol tended to have a relatively weak effect on mRNA expression compared with protein secretion, its combination with theophylline had a strong inhibitory effect on mRNA expression for both cytokines, the extent of which was practically comparable to that seen with dexamethasone. This suggests that the interaction between theophylline and β2 agonists is, at least in part, exerted at the level of mRNA expression of the cytokines. Although the 30 min pre-incubation time was considered sufficient for dexamethasone and the other drugs to initiate their genomic actions, their continued presence during the subsequent 24h of stimulation ensured that such effects were persistent and prolonged.

Unlike the β2 agonists whose cytokine-inhibitory and enhancing effects appeared to be dependent on intracellular cAMP, the effect of theophylline was clearly independent of this second messenger. Although theophylline is known to be a weak PDE inhibitor, this effect is usually seen at much higher concentrations of the drug. At the concentration of 50 µM used in most of the current study, it is unlikely that the effect is related to PDE inhibition/cAMP elevation. Indeed at concentrations up to 75 µM, theophylline produced no change in monocyte cAMP levels (data not shown).
Furthermore, with respect to IL-6 generation, its effect was completely opposite to those produced by agents that raise or mimic intracellular cAMP. Given that TNF-α and IL-6 have been reported to be involved in the pathophysiology of asthmatic inflammation (Broide et al., 1992; Konno et al., 1996; Barnes et al., 1998; Thomas, 2001), the tendency for β2 agonists to differentially affect the release of these cytokines is bound to be of therapeutic significance. It entails that any beneficial anti-inflammatory effect that these drugs might have by virtue of inhibiting the release of some inflammatory cytokines, such as TNF-α, is likely to be compromised by the concurrent enhancement of the release of other inflammatory cytokines like IL-6 and IL-8 (Kavelaars et al., 1997; Shames et al., 2001). This may, in fact, be part of the reason why the clearly demonstrable in vitro anti-inflammatory effect of short-acting β2-agonists is often inapparent clinically, unlike theophylline which inhibits both cytokines groups.

In view of the observed useful interaction between theophylline and β2 agonists on the release of pro-inflammatory cytokines, it is conceivable that when used together in the treatment of bronchial asthma, theophylline may be able to prevent the pro-inflammatory actions of the β2 agonists while complementing their anti-inflammatory actions. This would, of course, be in addition to the bronchodilatory effects of both drugs. In fact, data from the few clinical studies in which theophylline was combined with β2 agonists support our findings. In a randomized double blind cross-over trial in 30 patients with COPD, Tandon and Kailis (1991), showed that the combination of terbutaline and theophylline was superior to either drug alone in improving air flow. More recently, Vatrell et al., (2005) have also reported similar findings with a combination of salmeterol and theophylline in patients with moderate to severe asthma. Since the sizes of these studies were small, and the specific indices of inflammation were not measured, bigger and better-designed clinical studies are required to confirm these observations.
Our results also showed that the effect of theophylline was very similar to that of the steroid dexamethasone, both in the latter’s ability to inhibit the release of both TNF-α and IL-6, as well as in the interaction with salbutamol. This suggests that theophylline may have a potential steroid-sparing effect when used with β2 agonists in the treatment of asthma. The mechanistic basis of such steroid-like effect (both in the inhibition of cytokine release and interaction with cAMP-elevating agents) is not clear, but could indicate the involvement of a common repressive mechanisms. Of the many proposed mechanisms of action of theophylline such as PDE inhibition, increase in cytosolic Ca2+ and increased apoptosis (Yasui et al., 2000), adenosine receptor antagonism (Holgate et al, 1984), inhibition of PI3K (Foukas et al., 2002), inhibition of NF-κB (Umeda et al., 2002) and the induction of HDAC activity (Ito et al., 2002), perhaps only the last four may reasonably be expected to operate at concentrations of theophylline less than 50 µM. Although the current study was not designed to determine the mechanism of action and interaction of theophylline with β2 agonists or steroids, nevertheless, since the effect of theophylline was clearly genomic and significant at concentrations of 30-50 µM, it is more likely that the gene-dependent mechanisms especially, inhibition of PI3K, inhibition of NF-κB and induction of HDAC are involved, perhaps with histone acetylation/deacetylation as the point of convergence. It is well known that the activation of both PI3K and NF-κB leads to increased histone acetyltransferase (HAT) activity and that the inhibition of PI3K restores HDAC activity in smoke-induced airway inflammation in mice (Marwick et al., 2009), which would be consistent with the action of theophylline.

Given the above scenario, as well as the fact that the mechanism of anti-inflammatory action of steroids involves the recruitment of HDAC-2 activity, it can be argued that the basis of the additive interaction between theophylline and steroids is likely to involve co-operative actions whereby the former induces HDAC-2 activity that is recruited by the latter.

The induction of HDAC activity by theophylline may also contribute to its ability to antagonize β2 agonist-induced, cAMP-dependent enhancement of IL-6 release. Interleukin-6 is one of the
cytokines that are positively regulated by cAMP since the promoter region of its gene contains a cAMP-response element (CRE). By increasing intracellular cAMP, β₂ agonists cause the activation of cAMP-dependent protein kinase A (PKA). The latter phosphorylates the transcription factor CRE-binding protein (CREB) (Bartsch et al., 1998) to switch on the IL-6 gene transcription. Thus, by inducing HDAC activity, theophylline could potentially block the action of phosphorylated CREB and consequently IL-6 production. It has been shown that increased HDAC activity can switch off certain genes activated by phosphorylated CREB (Fass et al., 2003).

In conclusion, our results reveal that at clinically-relevant concentrations, theophylline is an effective inhibitor of the release of both TNF-α and IL-6 from human monocytes whereas the short-acting β₂ agonists inhibit the release of TNF-α, but enhance that of IL-6. More importantly, when used together with β₂-agonists, theophylline is able to suppress the undesirable pro-inflammatory effects of β₂ agonists - a property shared with steroids. If this effect is confirmed clinically, it is likely to lead to a scenario whereby theophylline plus β₂ agonists may be considered a possible alternative to steroid plus β₂ agonists in the treatment of asthma.
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Foot Notes

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Legends

Fig. 1: Effect of salbutamol and procaterol on the release of TNF-α (A) and IL-6 (B) in human monocytes stimulated with LPS (250 ng/ml). Cells were pre-incubated with the drugs for 30 min before stimulation. The amounts of the cytokines produced over 24 h by the control (un-stimulated) cells were in the ranges of 0.02 - 0.06 ng/10^6 cells and 0.5 – 0.8 ng/10^6 cells for TNF-α and IL-6, respectively. The corresponding values for LPS (250 ng/ml)-stimulated, but un-treated cells were 0.3 – 2.5 ng/10^6 cells and 3.2 – 10.5 ng/10^6 cells, respectively. Values are mean ± s.e.m for 6 experiments. *p<0.05; **p<0.01.

Fig. 2: Effect of agents that raise intracellular cAMP (PGE₂, forskolin and db-cAMP) on the release of TNF-α (A) and IL-6 (B) from monocytes stimulated with LPS (250 ng/ml). Cells were pre-treated with the drugs for 30 min before stimulation. Values are mean ± s.e.m for 5 experiments. *p<0.05; **p<0.01; ***p<0.01 compared with control (LPS alone).

Fig. 3: Effect of theophylline on the release of TNF-α (A) and IL-6 (B) in human monocytes stimulated with LPS (250 ng/ml). Cells were incubated with the drugs for 30 min before stimulation. Values are mean ± s.e.m, n=8. *p<0.05; **p<0.01; ***p<0.001 compared with control (LPS alone).
Fig. 4: Interaction of theophylline with salbutamol on the release of TNF-α (A) and IL-6 (B) in monocytes stimulated with LPS (250 ng/ml). Cells were pre-incubated with the drugs for 30 min before stimulation. Values are means ± s.e.m for 5 experiments. *p<0.05; **p<0.01; ***p<0.001 compared with control (LPS alone).

Fig. 5: Interaction between theophylline and PGE₂ (A) and between theophylline and db-cAMP (B) on LPS-induced IL-6 production in monocytes. Cells were pre-incubated for 30 min with the drugs before stimulation. Values are means ± s.e.m for 5 experiments. **p<0.01; ***p<0.001, compared with control (LPS alone).

Fig 6: Effect of the degree of stimulation on the actions and interactions of salbutamol (1 µM) and theophylline (50 µM) on LPS-induced release of TNF-α (A) and IL-6 (B). Cells were incubated with the drugs either alone or in combination for 30 min before stimulation. Values are means ± s.d. of triplicate wells of one experiment representative of two others of similar results.

Fig. 7: Dose-response relationship of dexamethasone on LPS-induced TNF-α (A) and IL-6 (B) and its interaction with salbutamol (C & D). Cells were incubated with the drugs either alone or in combination for 30 min before stimulation. Values are means ± s.e.m, for 5-7 experiments. *p<0.05; **p<0.01; ***p<0.001.

Fig. 8: The effect of theophylline and salbutamol (alone and in combination) on the expression of mRNA for TNF-α (A & B) and IL-6 (C & D) in LPS-stimulated monocytes. Gel figures (A & C) are representative, while histograms (B & D) are means ± s.e.m, for 4 experiments. Cells were
incubated with the drugs for 30 min before being stimulated for 2 h. *p<0.05, **p< 0.01; ***p<0.001.
Fig. 2

(A) 

![Graph showing TNF-\(\alpha\) (% Control) with bars and error bars for different concentrations of PGE\(_2\), Forskolin, and db-cAMP.](image)

(B) 

![Graph showing IL-6 (% Control) with bars and error bars for different concentrations of PGE\(_2\), Forskolin, and db-cAMP.](image)
Fig 5.

(A) IL-6 (% Control)

- C
- PGE\(_2\) (0.1 μM)
- Theo (50 μM)
- PGE\(_2\) + Theo

(B) IL-6 (% Control)

- C
- db-cAMP (50 μM)
- Theo (50 μM)
- db-cAMP + Theo

** and *** indicate statistical significance.
Fig. 7.

(A) TNF-α (% Control) vs. Log [Dexamethasone (M)]

(B) IL-6 (% Control) vs. Log [Dexamethasone (M)]

(C) TNF-α (% Control) for Control, Salb. (1.0 μM), Dexa (0.1 μM), and Salb + Dexa

(D) IL-6 (% Control) for Control, Salb. (1.0 μM), Dexa (0.1 μM), and Salb + Dexa
Fig. 8

(A)  

<table>
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<th>Treatment</th>
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<th>3</th>
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<tr>
<td>Dexa (0.1 µM)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Salb (1 µM)</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>Theo (50 µM)</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>LPS (1 µg/ml)</td>
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</tbody>
</table>

(B)  

![Graph showing TNF-α mRNA (TNF-α/β-Actin) levels](graphic)

(C)  

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<tr>
<td>Dexa (0.1 µM)</td>
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<td>-</td>
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</tr>
<tr>
<td>Salb (1 µM)</td>
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<td>+</td>
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<tr>
<td>Theo (50 µM)</td>
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<tr>
<td>LPS (1 µg/ml)</td>
<td>+</td>
<td>+</td>
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</tbody>
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(D)  

![Graph showing IL-6 mRNA (IL-6/β-Actin) levels](graphic)

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