Interactions between Theophylline and Salbutamol on Cytokine Release in Human Monocytes

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ABSTRACT

The combination of β2-adrenoceptor agonists (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 regard 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 compare it with a similar interaction between dexamethasone and salbutamol. Purified monocytes from normal donors were pretreated with the drugs (alone or in combination) and stimulated with lipopolysaccharide for 24 h. Released tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and their corresponding mRNA expressions, were determined and analyzed. Salbutamol (≥ 0.1 μM) significantly inhibited the release of TNF-α, but also significantly enhanced that of IL-6. In contrast, theophylline (50 μM) and dexamethasone (0.1 μM) strongly inhibited the generation of both cytokines. It is noteworthy that when the drugs were 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 they were combined with clinically relevant concentrations of theophylline, theophylline, like dexamethasone, was capable of augmenting the anti-inflammatory effects of the β2-agonists while at the same time preventing their proinflammatory effect. Thus, theophylline may have a potentially useful steroid-sparing effect.
(Holgate et al., 1984; Yasui et al., 2000), inhibition of nuclear factor-xB (NF-xB) (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 an 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 proinflammatory 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 proinflammatory 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 proinflammatory 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 a 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, because they seem to act in a complementary, and sometimes synergistic, manner (Barnes, 2006). Theophylline has also been combined with inhaled steroids or antileukotriene drugs with significantly better clinical outcomes (Ukena et al., 2002). 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 theophylline and salbutamol.

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

Materials and 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 Kuwait University’s Institutional Ethics Committee. Mononuclear cells were first isolated from heparinized blood by the gradient centrifugation method. Highly purified monocytes were then isolated immunomagnetically with the Monocyte Negative Isolation kit (Invitrogen, Carlsbad, CA). They were then plated at a concentration of 5 x 10⁵ cells/ml in a 48-well plate, preincubated with the drugs (salbutamol, procaterol, theophylline, dexamethasone, dibutylryl cAMP (dbcAMP), forskolin, and 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 24 h 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 with 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 method using assay kits supplied by R&D Systems (Minneapolis, MN) and following the manufacturer’s instructions.

Determination of Cytokine mRNA Expression. The method used was based on a previously published protocol (Abu-Ghefreh et al., 2009). In brief, purified monocytes were adjusted to a concentration of 10⁵ viable cells/ml, and 2-ml aliquots were added to each well of a six-well plate (2 x 10⁶ cells/well). Cells were preincubated 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 with a Nucleospin RNA II kit (Machery-Nagel, Düren, Germany) according to the manufacturer’s instructions. The extracted RNA (1 µg) was reverse-transcribed by using the High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) and then amplified by semiquantitative reverse transcription-polymerase chain reactions. The sequences of the oligonucleotide primers were as follows: TNF-α, 5'-GTCGACACCTCGAGCAGAC-3'; IL-6, 5'-ATGAAGCTCTTCTCCACAGCGC-3'; 5'-GAAGAGCTCTCAGCTGTCGGCC-3' and 5'-AACGAGCTGCTGTCGGCCCTGAG-3'; β-actin, 5'-CGGAGGAAAAGCCCCTGGCAGG-3'; 5'-TGACGAGGGGACCGGCGACGG-3' and 5'-TCACCCACACTTGCTGCCCCATCTACAGA-3'. Thermocycling was carried out according to the following profile: 95°C for 5 min before the first cycle, 94°C for 30 s, annealing temperature (61°C for TNF-α, 65°C for IL-6, and 64°C for β-actin) for 30 s and 72°C for 30 s repeated for 30 cycles followed by a final extension at 72°C for 5 min.

The amplified products were then electrophoresed through a 1.5% agarose gel at 100 V, and the gel was stained with ethidium bromide. The density of each band was quantified in a densitometer, using the Alpha Imager Gel Documentation System (Alpha Innotech, San Leandro, CA). 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, 628, and 294 base pairs, 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 (IC₅₀ values) or enhancement of response (EC₅₀ values) were calculated from the concentration-effect curves by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA). Differences between mean values were analyzed either by one-sample t test or analysis of variance 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 Fig. 1A, pretreatment of human monocytes with the β₂-agonists salbutamol and procaterol resulted in a significant and concentration-dependent inhibition of LPS-induced TNF-α release over 24 h. The mean (95% confidence interval) of the concentrations achieving 50% of maximal inhibition (IC₅₀ values) were 31.6 (17.3–52.2) 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 pro-
caterol, respectively. In contrast, both drugs significantly
enhanced the concurrently released IL-6 in the same concen-
tration range at which they suppressed TNF-α and IL-6, respectively. The corresponding values for LPS (250 ng/ml)-stimulated, but untreated, cells were 0.3 to 2.5 and 3.2 to 10.5 ng/10^6 cells, respectively. Values are mean ± S.E.M. for six experiments.* p < 0.05; ** p < 0.01.

Because these drugs are known to act by increasing intra-
cellular cAMP (Bailly et al., 1990), their effects were com-
pared with those of other agents that raise intracellular

cAMP, namely PGE_2 (which, like β_2-agonists, is a G_s-coupled

receptor agonist), forskolin (a direct activator of adenylyl
cyclase), and db-cAMP (a cell-permeant cAMP analog). As
shown in Fig. 2, all three drugs, to varying degrees, sig-
nificantly 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 of
≥30 and 100 μM; the release of both cytokines was com-
pletely abolished. In the concentration range of 30 to 50 μM,
which is marginally below the typical bronchodilatory
plasma concentration of the drug (55–110 μM) (Barnes and
Pauwels, 1994) theophylline achieved 45 to 60% inhibition of
both responses.

Interaction of Theophylline with β_2-Agonists on the
Release of TNF-α and IL-6. In view of the finding that
β_2-agonists enhance, whereas theophylline inhibits, IL-6 se-
cretion in LPS-stimulated monocytes, it was of interest to
determine the effect of the two drugs used in combination. As shown in Fig. 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 whether 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 Fig. 5, 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 seems 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 whether 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 Fig. 6, the differential effect of salbutamol on the release of TNF-α and IL-6 and its interaction with theophylline were similar at the two levels of stimulation and 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 seemed 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. After 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 whether steroids, which are the mainstay of the anti-inflammatory therapy of asthma, exhibited a similar interaction with β2-agonists. As shown in Fig. 7A, dexamethasone inhibited LPS-induced release of TNF-α with a high potency (IC50/H9251 = 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 dexamethasone was added with salbutamol, the inhibitory effects of the two drugs on TNF-α release were essentially additive (Fig. 7C), whereas the IL-6-enhancing effect of salbutamol was lost (Fig. 7D). Thus, dexamethasone seems to behave in a very similar manner as theophylline when combined with a β2-agonist.

To explore the mechanism by which theophylline acts and interacts with β2-agonists, the effect of the drug, either alone or combined with salbutamol, on the expression of mRNA for TNF-α and IL-6 was studied. As shown in Fig. 8, A and B, pretreatment 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 the two drugs were used in combination, their effects were essentially additive. On the other hand, salbutamol increased the expression of mRNA for IL-6 (although it did not reach statistical significance), whereas theophylline significantly suppressed the expression of mRNA for IL-6 (Fig. 8, C and D). When the drugs were 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 of salbutamol and dexamethasone. The results obtained showed 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 proinflammatory mediators, which were largely independent of the degree of cytokine induction, seemed to depend on the generation of intracellular cAMP, because 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 (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/liter) (Barnes and Pauwels, 1994; Ohta et al., 2004). Recent reports have shown that low doses of 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 because at 30 to 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.

Fig. 7. Dose-response relationship of dexamethasone (Dexa) on LPS-induced TNF-α (A) and IL-6 (B) and its interaction with salbutamol (Salb) (C and D). Cells were incubated with the drugs either alone or in combination for 30 min before stimulation. Values are means ± S.E.M. for five to seven experiments. , p < 0.05; , p < 0.01; , p < 0.001.
iology of asthma (Gosset et al., 1991; Broide et al., 1992; Barnes et al., 1998; Thomas, 2001).

The combination of a clinically relevant concentration of theophylline (50 μM) with salbutamol (0.1–1.0 μM) produced an interesting result. Although 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 et al. (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 with 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 preincubation time was considered sufficient for dexamethasone and the other drugs to initiate their genomic actions, their continued presence during the subsequent 24 h of stimulation ensured that such effects were persistent and prolonged.

Unlike the β₂-agonists whose cytokine-inhibitory and -enhancing effects seemed to depend 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;
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In view of the observed useful interaction between theophylline and β2-agonists on the release of proinflammatory cytokines, it is conceivable that when they are used together in the treatment of bronchial asthma theophylline may be able to prevent the proinflammatory 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 crossover trial in 30 patients with chronic obstructive airway disease, Tandon and Kailis (1991) showed that the combination of terbutaline and theophylline was superior to either drug alone in improving air flow. More recently, Vatrella et al. (2005) have also reported similar findings with a combination of salmeterol and theophylline in patients with moderate to severe asthma. Because the sizes of those studies were small, and the specific indices of inflammation were not measured, bigger and better-designed clinical studies are required to confirm those observations.

Our results also showed that the effect of theophylline was very similar to that of the steroid dexamethasone, in both the latter’s ability to inhibit the release of both TNF-α and IL-6 and 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 a steroid-like effect (in both the inhibition of cytokine release and interaction with cAMP-elevating agents) is not clear, but could indicate the involvement of 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, because the effect of theophylline was clearly genomic and significant at concentrations of 30 to 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 activity and 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, and 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 cooperative 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 because the promoter region of its gene contains a cAMP-response element. By increasing intracellular cAMP, β2-agonists cause the activation of cAMP-dependent protein kinase A. The latter phosphorylates the transcription factor cAMP-response element-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 proinflammatory 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 combined with β2-agonists may be considered a possible alternative to steroids combined with β2-agonists in the treatment of asthma.

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References


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