Theophylline suppresses the release of tumour necrosis factor- α by blood monocytes and alveolar macrophages

M. Spatafora*, G. Chiappara⁺, A.M. Merendino*, D. D'Amico⁺,
V. Bellia*, G. Bonsignore*⁺

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ABSTRACT: The purpose of this study was to evaluate the effect of theophylline on tumour necrosis factor- α (TNF- α) release by human blood monocytes (BMo), and rat BMo and alveolar macrophages (AM).

BMo and AM were incubated in the absence or presence of theophylline, and the cell-free supernatants were harvested and tested for TNF- α activity by bioassay.

Theophylline dose-dependently reduced TNF- α release by human BMo: significant inhibition was observed at 100 μ M ($41 \pm 5.9\%$ of controls) and at 50 μ M ($59 \pm 4.8\%$ of controls), while the inhibitory activity of theophylline at 10 μ M ($71 \pm 8.9\%$ of controls) was not statistically significant. This activity was maximal at 2 h and declined at 4 h ($59 \pm 5.2\%$ of controls) and 24 h ($89 \pm 3.1\%$ of controls). Northern analysis performed on ribonucleic acid (RNA) extracted from human BMo demonstrated that theophylline was able to reduce TNF- α gene expression. Comparable levels of inhibition of TNF- α release were observed on rat BMo and AM (rat BMo $42 \pm 4.9\%$ of controls; rat AM $38 \pm 1.7\%$ of controls), suggesting that BMo and AM are equally susceptible to suppression of TNF- α release induced by theophylline.

These results indicate that theophylline suppresses TNF- α release by mononuclear phagocytes. Since TNF- α is involved in the pathogenesis of bronchial hyper-responsiveness and asthma, our results suggest that the therapeutic activity of theophylline might be partly related to its effect on TNF- α release.

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*Istituto di Medicina Generale e Pneumologia, Università degli Studi, Palermo, Italy. ⁺Istituto di Fisiopatologia Respiratoria, Consiglio Nazionale delle Ricerche, Palermo, Italy.

Correspondence: M. Spatafora
Istituto di Medicina Generale e Pneumologia
Università degli Studi
Via Trabucco
180 - 90146 Palermo
Italy

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Theophylline (1,3 dimethylxanthine) is one of the most widely-used drugs in the therapy of bronchial asthma and chronic obstructive pulmonary disease. The therapeutic activity of theophylline has been classically related to its bronchodilator effect [1], and to its ability to improve diaphragmatic contractility [2]. However, it is now being increasingly recognized that theophylline is able to suppress, both *in vitro* and *in vivo*, a variety of inflammatory and immune cell functions. Previous studies have shown that theophylline reduces the phagocytic and bactericidal capabilities of polymorphonuclear leucocytes [3] and macrophages [4], the release of reactive oxygen species by macrophages [5], the proliferative responses of T-lymphocytes to mitogens [6], and the cytotoxic activity of natural killer cells [7]. Since inflammatory processes may play a crucial role in the development of bronchial obstruction, it is thus conceivable that the therapeutic activity of theophylline is related, at least partly, to its effects on inflammation.

Tumour necrosis factor- α (TNF- α), a 17 kDa cytokine produced by lipopolysaccharide (LPS)-stimulated mononuclear phagocytes [8], is an important mediator of inflammation [9, 10]. The release of TNF- α by mononuclear phagocytes is regulated by the intracellular levels of cyclic

adenosine 3':5' monophosphate (cAMP). Exogenous cAMP analogues [11] and agents, such as prostaglandin E₂ (PGE₂) capable of increasing the intracellular level of cAMP, reduce the release of bioactive TNF- α by down-regulating the expression of TNF- α gene [12, 13].

Since theophylline increases the intracellular accumulation of cAMP by competitive inhibition of the phosphodiesterase enzyme [14, 15], we investigated the effects of theophylline on TNF- α release by human peripheral blood monocytes (BMo). In previous studies, alveolar macrophages (AM) were shown to be less susceptible than BMo to PGE₂-mediated suppression of TNF- α release [16], therefore, we also studied the effects of theophylline on TNF- α release by AM.

Materials and methods

Reagents and culture materials

Complete medium consisted of RPMI 1640 + 1% foetal calf serum + L-glutamine (2 mM) + penicillin (100 U·ml⁻¹) + streptomycin (100 μ g·ml⁻¹) (all from Gibco, Paisley,

UK). Theophylline (1,3 dimethylxanthine) was purchased from Sigma (St. Louis, MO, USA), dissolved in phosphate buffered saline (PBS) (Gibco) and further diluted by adding complete medium to the stock solution. Human recombinant (HR)-TNF- α (lot EB 6012; specific activity 5×10^6 U·mg⁻¹ protein) and monoclonal anti-TNF antibody (No. 199; isotype IgG1, K) were generously provided by Knoll (Ludwigshafen, Germany). All reagents were negative for endotoxin contamination, as assessed by Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA, USA).

Isolation of human BMo

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation of normal donors' buffy coats. BMo were enriched by fractionation of mononuclear cells on a one-step Percoll (Pharmacia) gradient, as described in detail previously [17]. These procedures allowed the recovery of a virtually pure population of BMo, as assessed by morphology and nonspecific esterase staining of cyto-centrifuge preparations.

Isolation of rat AM and BMo

Adult, pathogen-free, male Wistar rats (Charles River, Calco, Italy), weighing 200–300 g, were used. The animals were maintained in pathogen free colonies with standard laboratory feed and water *ad libitum*.

Rat AM were obtained by bronchoalveolar lavage (BAL). The animals were sacrificed by intraperitoneal injection of 2–5 ml of urethane (Sigma) at a concentration of 250 mg·ml⁻¹. The chest was opened and the trachea was cannulated with polypropylene tubing secured with a ligature. The lungs were lavaged *in situ* with a total of 50 ml of 0.9% NaCl solution, in 10 aliquots of 5 ml each. The retrieved BAL fluid was filtered through a layer of sterile gauze and centrifuged at 400×g for 10 min. The cell pellets from 4–8 animals were pooled, washed twice in PBS and counted with a haemocytometer. The rat AM preparation was always more than 95% pure, as judged by morphology on Diff-Quick (Merz-Dade, Duding, Switzerland) stained cytocentrifuge preparations.

Rat peripheral BMo were isolated from blood obtained by puncture of the inferior vena cava, using the same methodology as used for the isolation of human BMo (see above). Typically, blood samples from 6–10 rats had heparin added and were pooled before the beginning of purification procedures.

Evaluation of TNF- α release

Human BMo and rat AM and BMo were resuspended in complete medium at a concentration of 1×10^6 cells·ml⁻¹, in the absence or presence of 1 μ g·ml⁻¹ LPS from *Escherichia coli* (serotype 0111:B4, Sigma) and/or theophylline at the appropriate concentration. After incubation (37°C, 5%

CO₂), culture tubes were centrifuged (600×g, 10 min) and lytic supernatants were harvested and frozen at -70°C until assayed for bioactive TNF- α .

TNF- α activity was measured in culture supernatants by a specific cytotoxicity assay, using as targets actinomycin D (Sigma) treated WEHI 164 murine sarcoma cells, as described previously [17]. The cytotoxicity of BMo culture supernatants on TNF- α sensitive target cells was assessed by the colorimetric assay as described by MOSMANN [18], using as substrate the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma). This method is based on the ability of living cells to reduce the yellow MTT substrate to a blue formazan compound. Briefly, target cells were incubated (37°C, 5% CO₂) in 96-well flat-bottomed plates with culture supernatants or serial dilutions of a standard HR-TNF- α solution. After 20 h, an MTT solution was added to the test wells to give the final MTT concentration of 50 μ g·ml⁻¹ and, after an additional 4 h, the assay plates were centrifuged and the pelleted formazan crystals were solubilized with dimethyl sulphoxide (Farmitalia Carlo Erba, Milan, Italy). Absorbances were read at 540 nm by using an automated microplate reader (Multiscan MCC 340, Flow, Milan, Italy) and cytotoxic indices (CI) were calculated as follows: CI (%) = $(1 - A/B) \times 100$, where A is the optical density (OD) 540 in the test wells and B is the OD 540 in control wells containing complete medium instead of lytic supernatants. TNF- α activity, expressed as U·ml⁻¹, was quantified in lytic supernatants by interpolation on HR-TNF- α standard curve. All tests were run in quadruplicate and variation never exceeded 12%. The specificity of this bioassay was repeatedly checked by testing the ability of anti-TNF- α antibody to block cytotoxicity. The sensitivity was 0.1 U·ml⁻¹.

To verify that theophylline did not influence the bioassay by modifying the sensitivity of target cells to TNF- α -mediated lysis, theophylline (100 μ M) was added to target cells before delivery of serially diluted HR-TNF- α . After 20 h (37°C, 5% CO₂), the cytotoxicity was determined and compared to that exerted by the same concentrations of HR-TNF- α on target cells without addition.

Northern analysis

To evaluate the expression of TNF- α gene, a Northern analysis was carried out on LPS-stimulated BMo (1 μ g·ml⁻¹, 2 h) in the absence or presence of theophylline (100 μ M). Ribonucleic acid (RNA) was extracted using guanidine isothiocyanate 4 M and purified by a cesium chloride gradient, as described previously [19]. The amount of RNA was measured by spectrophotometrical analysis, and 10 μ g of each RNA sample was electrophoresed in a 1% denaturing agarose gel and alkali blotted onto nylon membranes (Hybond-N⁺, Amersham, UK) as recommended by the manufacturer. The filters were then hybridized with a complementary deoxyribonucleic acid (cDNA) TNF- α probe labelled with ³²P-dCTP (deoxycytidine triphosphate) (Amersham).

The probe was obtained by polymerase chain reaction (PCR) amplification of first-strand random primed cDNA synthesized using 25 μ g of total RNA extracted from human BMo stimulated with phorbol myristate acetate (PMA) (Sigma) [20]. The open reading frame (ORF) of the TNF- α gene was PCR-amplified, using 1 μ m of the random-primed cDNA reaction and TNF- α specific primers, which were synthesized using the System 200A DNA Synthesizer (Beckman, Fullerton, CA, USA). These primers (5'-GGAAAGGACACCATGAGCACT-3', sense; and 5'-GAAGGTTGGATGTTTCGTCCTC-3', antisense) were designed on the basis of the published sequence of the human TNF- α gene [21]. The cDNA PCR-amplification consisted of 35 cycles (each cycle of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min). The resulting PCR fragment of 736 bp (corresponding to the expected size of the TNF- α ORF) was separated by agarose gel electrophoresis and purified using the GeneClean kit (Bio 101, La Jolla, CA, USA).

The TNF- α probe was 32 P-dCTP-labelled using the Random primers DNA-labelling system (Bethesda Research Laboratories, Gaithersburg, MD, USA). After overnight incubation the filters were washed and exposed for autoradiography to X-Omat XAR2 (Kodak, Rochester, NY, USA) films at -80°C for 10 days before developing. The autoradiographs were quantitated using laser scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA, USA). Equivalent amounts of total RNA $\cdot$ well $^{-1}$ were assessed by ethidium bromide staining of 28S and 18S ribosomal RNA.

Statistical analysis

The data are reported as mean $\pm$ SEM. Comparisons were made by the analysis of variance (ANOVA)-based Fisher protected least significant difference test. A value of $p < 0.05$ was considered as significant.

Results

Activity of theophylline on TNF- α production by LPS-stimulated BMo

Human peripheral BMo were incubated in the absence or presence of serial theophylline concentrations, and the release of TNF- α at 2 h was quantified by a specific bioassay. While TNF- α production by unstimulated BMo was below the sensitivity limit of the bioassay (data not shown), the release of TNF- α by LPS-stimulated BMo was 376.8 ± 130.5 U $\cdot$ ml $^{-1}$. Theophylline suppressed TNF- α release in a dose-dependent fashion (fig. 1): the highest inhibition was observed at 100 μ M ($41 \pm 5.9\%$ of controls; $p < 0.01$), and significant inhibition ($59 \pm 4.8\%$ of controls; $p < 0.05$) was also observed at 50 μ M, a concentration comparable to the *in vivo* therapeutic levels of theophylline in the blood [15]. The inhibitory activity of theophylline at the concentration of 10 μ M ($71 \pm 8.9\%$ of controls) was not statistically significant.

Fig. 1. – Effect of theophylline on TNF- α release by LPS-stimulated human BMo. After purification procedures, BMo were stimulated with LPS (1μ g $\cdot$ ml $^{-1}$) in the absence or presence of theophylline at serial concentrations. The release of TNF- α at 2 h was assessed by a specific bioassay. Data represent mean $\pm$ SEM of five separate experiments. $\square$: control; $\blacksquare$: theophylline. TNF- α : tumour necrosis factor- α ; LPS: lipopolysaccharide; BMo: blood monocytes.

Theophylline had no influence on the TNF- α bioassay, and did not modify the sensitivity of target cells to TNF- α -mediated lysis. The addition of theophylline at the highest suppressive concentration (100 μ M) to target cells did not modify the CI of HR-TNF- α (fig. 2), thus indicating that theophylline has no effects on the susceptibility of target cells to HR-TNF- α over the conditions used in the bioassay.

Fig. 2. – Effect of theophylline on the susceptibility of actinomycin-D treated WEHI 164 cells (target cells) to HR-TNF- α mediated cytotoxicity. Theophylline (100 μ M) was added to target cells before delivery of serially diluted HR-TNF- α and cytotoxicity was measured at 20 h. Data represent mean $\pm$ SEM of three separate experiments. $\square$: control; $\blacksquare$: +theophylline 100 μ M. HR-TNF- α : human recombinant tumour necrosis factor- α .

Fig. 3. — Kinetic analysis of theophylline-induced inhibition of TNF- α release by human BMo. LPS-stimulated BMo were cultured for 2, 4 and 24 h in the absence (control) or presence of theophylline (100 μ M) and the inhibition of TNF- α release was calculated at each time-point. Data represent mean $\pm$ SEM of five separate experiments. For abbreviations see legend to figure 1.

Time-course analysis of theophylline-induced inhibition of TNF- α release

The inhibition provided by theophylline on TNF- α release by LPS-stimulated BMo was calculated at 2, 4, and 24 h. As shown in figure 3, the inhibition of TNF- α release by theophylline was maximal at 2 h, while reduced levels of inhibition were observed at subsequent time-points (4 h 59 $\pm$ 5.2% of controls; 24 h 15 $\pm$ 3.1% of controls). In addition, since it was previously demonstrated that the inhibitory activity of theophylline on immune cell functions strictly depends on the continuous presence of the drug in the cultures [6], we sought to determine whether removal of theophylline from complete medium altered its inhibitory activity on TNF- α release. Accordingly, after preincubation for 2 h with theophylline (100 μ M), BMo were repeatedly washed, incubated with LPS and the release of TNF- α at 2 h was measured. In three consecutive experiments, removal of theophylline after preincubation completely abrogated the inhibitory activity on TNF- α release (table 1), demonstrating that the continuous presence of the drug is mandatory for suppression of TNF- α release.

Table 1. — Effect of preincubation with theophylline (2 h; 100 μ M) on LPS-induced release of the TNF- α by human BMo

Expt No.	Control	Theophylline	
1	144.2 $\pm$ 9.8	144.8 $\pm$ 7.9	NS
2	43.8 $\pm$ 10.8	40.0 $\pm$ 10.8	NS
3	526.7 $\pm$ 24.3	530.8 $\pm$ 50.5	NS

Data are presented as mean $\pm$ SEM of TNF- α activity (U·ml⁻¹). After preincubation for 2 h with theophylline (100 μ M) or control complete medium, BMo were repeatedly washed and incubated with LPS (2 h, 37°C, 5% CO₂). TNF- α activity in culture supernatants was quantified by the MTT bioassay. LPS: lipopolysaccharide; TNF- α : tumour necrosis factor- α ; BMo: blood monocytes; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

Fig. 4. — Graphical representation of laser densitometry of TNF- α mRNA accumulation in LPS-stimulated human BMo in the absence or in the presence of theophylline (100 μ M, 2 h). Shown as an insert is an autoradiograph of a representative Northern analysis. Theophylline was able to reduce LPS-induced accumulation of TNF- α mRNA in human BMo. mRNA: messenger ribonucleic acid; OD optical density. For further abbreviations see legend to figure 1.

Expression of the TNF- α gene by BMo

We next investigated the expression of the TNF- α gene by BMo using Northern analysis, with a ³²P-labelled cDNA probe. The results of the laser densitometry analysis of a representative experiment are shown in figure 4. Consistent with the results of the TNF- α bioassays, the autoradiograph shows that maximal intensity of the signal was observed in the lane containing RNA extracted from LPS-stimulated BMo, and reduced levels of intensity were observed in the lane containing RNA from LPS-stimulating BMo in the presence of theophylline (100 μ M). These results indicate that theophylline is able to reduce TNF- α messenger ribonucleic acid (mRNA) accumulation in LPS-stimulated human BMo.

Effects of theophylline on TNF- α release by normal AM

In previous studies, AM were shown to be relatively resistant to TNF- α -inhibitory factors [16]. However, when we compared the inhibitory activity of theophylline (100 μ M; 2 h) on TNF- α release by normal rat AM and BMo, we did not detect any difference (table 2). Hence,

Table 2. — Effect of theophylline (2 h; 100 μ M) on LPS-induced release of TNF- α by rat BMo and AM

	Control	+Theophylline	Inhibition %
Rat BMo	123.3 $\pm$ 50.7	50.8 $\pm$ 18.3	42 $\pm$ 4.9
Rat AM	146.1 $\pm$ 66.7	56.1 $\pm$ 25.1	38 $\pm$ 1.7

Data are presented as mean $\pm$ SEM of TNF- α activity (U·ml⁻¹) of three separate experiments. AM: alveolar macrophages. For further abbreviations see legend to table 1.

rat AM and BMo are equally susceptible to suppression of TNF- α release by theophylline.

Discussion

The results presented here show that theophylline inhibits TNF- α production by LPS-stimulated BMo and AM in a dose-dependent fashion, and at concentrations comparable to the *in vivo* therapeutic blood levels of the drug. This effect is rapid and transient, and requires the continuous presence of the drug in culture.

Our findings on human BMo confirm and extend previous data reported by ENDRES *et al.* [22], who demonstrated the ability of theophylline (and other phosphodiesterase inhibitors) to suppress TNF- α production by increasing the intracellular accumulation of cAMP. In that study, however, mononuclear cell suspensions containing 20–24% of BMo were used as TNF- α -releasing cells, and no information was available on the expression of TNF- α gene. Our studies, performed on highly purified preparations of human BMo, show that the inhibitory activity of theophylline on TNF- α production is related to inhibition of TNF- α gene expression. In this respect, our findings extend the results of STRIETER *et al.* [23], who demonstrated the ability of phosphodiesterase inhibitors to suppress TNF- α production and TNF- α gene expression by LPS-stimulated murine peritoneal macrophages, suggesting that cAMP is significantly involved in the regulation of TNF- α synthesis. Consistent with this hypothesis are the observations of RENZ *et al.* [24], who demonstrated that the release of TNF- α by rat peritoneal macrophages is reduced by cAMP and agents increasing the intracellular cAMP concentration. In addition, our studies suggest that differentiation processes of mononuclear phagocytes do not modify their susceptibility to regulation of TNF- α release provided by theophylline. In this respect, theophylline differs from PGE₂, a TNF- α -regulatory factor with a low activity on AM [16]. Although we are unable to explain this discrepancy, it is reasonable to hypothesize that, in addition to cAMP, other endogenous mediators are involved in the regulation of TNF- α release by AM, and that theophylline might also interact with the latter mediators. It cannot be excluded that this unknown additional mechanism may account for the observation that longer incubation with theophylline results in a lower inhibition of LPS-induced TNF- α release.

Since TNF- α is likely to be involved in the inflammatory processes associated with bronchial asthma, our results support the hypothesis that the therapeutic activity of theophylline might also be related to the inhibition of TNF- α production by mononuclear phagocytes. The inhalation of TNF- α has recently been shown to induce bronchial hyperresponsiveness and neutrophilic inflammation in the airways of normal rats [25]. These effects are fully reproduced by the inhalation of bacterial LPS, the major TNF- α -releasing factor [26]. In addition, inhaled LPS increases nonspecific bronchial responsiveness in asthmatic subjects [27]. LPS is widely distributed in the environment and contaminates domestic water, air humidifiers, household and organic dusts [25]. Therefore, although the involvement of other inflammatory media-

tors cannot be excluded, LPS-induced elicitation of TNF- α release by mononuclear phagocytes (and other inflammatory cells) located in the airways probably plays a major role in the pathogenesis of bronchial inflammation and hyperresponsiveness. In addition to its own inflammatory activities, TNF- α induces the release of other mediators, such as the powerful neutrophil chemotactic and activating factor, interleukin-8 [28]. Moreover, TNF- α increases neutrophil adherence to endothelial cells by increasing the expression of adhesion molecules on the surfaces of both interacting cells [29], and promotes the transendothelial passage of neutrophils [30]. Not only may TNF- α play an important role in the development of nonspecific bronchial inflammation and hyperreactivity, but also it may participate in the lung inflammatory processes that follow the inhalation of a specific antigen in allergic asthmatic patients. TNF- α is released by AM of allergic asthmatic patients after the development of late asthmatic reaction induced by allergen inhalation [31]. Moreover, immunoglobulin E (IgE) receptor triggering by specific antigens or anti-IgE antibodies induces, in addition to the release of histamine and arachidonic acid metabolites, the expression of the TNF- α gene and the release of bioactive TNF- α by rat basophils [32], and rat lung explants [33]. Hence, the suppressive activity of theophylline on TNF- α release might have important implications with regard to its therapeutic potential in nonspecific and allergic bronchial inflammation and hyperresponsiveness.

The hypothesis that the therapeutic activity of theophylline may be partly related to its anti-inflammatory properties comes from the observation that theophylline has a more pronounced protective effect on the late asthmatic reaction after allergen challenge than on the immediate reaction [34]. This concept is supported by the results of a study by MAPP *et al.* [35] on a model of late asthmatic reaction occurring after bronchial challenge with the occupational agent toluene diisocyanate (TDI). In that study, although theophylline did not modify bronchial hyperreactivity, it was able to significantly inhibit the late phase reaction. Moreover, in a more recent study by the same group, oral slow-release theophylline was able to inhibit the allergen-induced asthmatic reaction and the associated increase of bronchial responsiveness [36]. It is still not known whether these effects of theophylline are related to its suppressive activity on TNF- α release, and whether other mediators of inflammation are also involved.

In conclusion, we have described an *in vitro* inhibitory effect of theophylline on TNF- α release and TNF- α gene expression by mononuclear phagocytes. Further studies are in progress to evaluate the *in vivo* activity of the drug on this and other functions of inflammatory cells.

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