Tumour necrosis factor-\(\alpha\) induces hyperreactivity in tracheal smooth muscle of the guinea-pig \textit{in vitro}


**ABSTRACT:** Recent studies have implicated a role for tumour necrosis factor-\(\alpha\) (TNF\(\alpha\)) in the development of the asthmatic reaction. In this study, we examined the influence of TNF\(\alpha\) on isotonic contraction of tracheal smooth muscle of the guinea-pig \textit{in vitro} in response to methacholine.

Tracheal rings were incubated with recombinant human (rh)TNF\(\alpha\) (3\(\times\)10\(^{-11}\) M) for 30 min, and concentration-response curves to methacholine before and after incubation with rhTNF\(\alpha\) were compared with the control.

The present study demonstrates that rhTNF\(\alpha\) increases maximal isotonic contraction of tracheal smooth muscle to methacholine (mean\(\pm\)SEM 169.6\(\pm\)4.3\%, \(p<0.005\)). This effect was observed only after a 30 min delay between incubation and methacholine challenge testing. Experiments with 10\(^{-11}\)–10\(^{-8}\) M rhTNF\(\alpha\)-\(\alpha\) yielded similar results at all concentrations used. The effects of rhTNF\(\alpha\) (10\(^{-11}\) M) on tracheal hyperreactivity could be completely inhibited by coincubation with dimeric rTNF-receptor-p80 construct (10\(^{-6}\) M) (\(p<0.01\)). In order to analyse secondary mediator release, experiments using coincubation with indomethacin (10\(^{-4}\) M) and WEB 2086 (10\(^{-4}\) M), a specific platelet activating factor (PAF) antagonist, demonstrated that the effect of rhTNF\(\alpha\) on tracheal rings was mediated by PAF, since WEB 2086 completely inhibited rhTNF\(\alpha\)-induced hyperreactivity (\(p<0.05\)).

In conclusion, this study demonstrates that recombinant human tumour necrosis factor-\(\alpha\) induces hyperreactivity in tracheal smooth muscle \textit{in vitro}, which was shown to be mediated by platelet activating factor. Our study emphasizes the role of tumour necrosis factor-\(\alpha\) in the pathophysiology of bronchial hyperresponsiveness.


Bronchial hyperresponsiveness (BHR) is regarded as an important characteristic of asthma and correlates with the severity of the disease [1]. BHR can be induced after allergen inhalation in allergic asthmatics, after viral respiratory tract infections, industrial chemical inhalation and endotoxin inhalation [2]. In asthmatic patients, endotoxin (lipopolysaccharide (LPS)) primarily induces a fall in forced expiratory volume in one second (FEV\(1\)) and secondarily an increase in BHR. However, it is not certain that LPS itself is primarily responsible for inducing BHR. Tumour necrosis factor-\(\alpha\) (TNF\(\alpha\)) has been found to represent an important mediator of LPS-induced effects [3]. In animal models, TNF\(\alpha\) levels were elevated in both BAL fluid [5] and sputum [6] in the late asthmatic reaction and in both BAL and bronchial mucosa in symptomatic asthmatic patients [7, 8]. In occupational asthma, increased expression of TNF\(\alpha\) was observed in the bronchiolar submucosa of workers with toluene disocyanate-induced asthma [9]. Recently, TNF\(\alpha\) inhalation was shown to induce BHR in healthy human volunteers, supporting a primary role for TNF\(\alpha\) in the development of BHR [10].

TNF\(\alpha\) can be released by various cell types involved in the inflammatory response, like monocytes, alveolar macrophages, mast cells, neutrophils and T-lymphocytes, depending upon the stimulus used [11]. In stable asthmatics, increased release of TNF\(\alpha\) was observed in alveolar macrophages, both spontaneously and after stimulation [12]. In allergic asthmatics, mast cells have been shown to be a source for TNF\(\alpha\) [8], and mast-cell activation initiated by immunoglobulin (Ig)E-receptor cross-linking is considered to be a source for TNF\(\alpha\) release [13]. Furthermore, T-helper (Th)2 lymphocytes play an important role in the development of asthma and produce a wide array of cytokines like interleukin (IL)-4, IL-5, granulocyte macrophage colony stimulating factor (GM-CSF) and TNF\(\alpha\) [14]. In order to investigate whether TNF\(\alpha\) affects the constrictor response of bronchial smooth muscle directly, we investigated the influence of TNF\(\alpha\) on smooth muscle response to methacholine in guinea-pig tracheal strips. We additionally investigated whether secondary mediator release is involved in TNF\(\alpha\)-induced effects. This study demonstrates the ability of TNF\(\alpha\) to induce hyperreactivity in tracheal smooth muscle of the guinea-pig \textit{in vitro} and provides evidence for the involvement of platelet activating factor (PAF) in TNF\(\alpha\)-induced BHR.
Methods

Reagents

Acetyl-β-methylcholine chloride (methacholine chloride) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Indomethacin was obtained from OPG-pharma (Utrecht, The Netherlands) and diluted in dimethyl-sulphoxide. Recombinant human (rh) TNFα was a gift from Knoll/BASF (Ludwigshafen, Germany). Recombinant TNF-receptor-p80:Fc (rTNFR-p80:Fc) (a soluble human TNF-receptor (p80) linked to the Fc portion of human IgG1) was kindly provided by Immunex (Seattle, WA, USA) [15]. WEB 2086 [3-4-(chlorophenyl)-9-methyl-6H-thieno[3,2][1,2,4]triazolo[4,3-a][1,4]diazepine-2-yl]-[4-morpholinyl]-1-propanone), a specific PAF antagonist [16], was a gift from H. Heuer, Boehringer Mannheim GmbH (Mannheim, Germany). rhTNFα, rTNFR-p80:Fc and WEB 2086 were dissolved in sterile phosphate-buffered saline (PBS). All solutions were freshly prepared.

Tracheal preparation

Dunkin-Hartley guinea-pigs (300–400 g), (Harlan CPB, Zeist, The Netherlands) were sacrificed by cervical dislocation. Tracheas were rapidly dissected free from surrounding tissue, excised and opened in longitudinal direction opposite to the muscle layer by cutting through the cartilage. Tracheal strips (each containing one cartilage ring) were placed in a 20 mL organ bath containing Krebs buffer solution of 37°C (as described previously) [17] and continuously gassed with 95% O2 and 5% CO2 (pH 7.4).

Experimental design

Tracheal smooth muscle response was measured isotonic with a passive weight of 0.5 g in all experiments. After an equilibration period of 60 min with four intermediate changes of buffer solution, cumulative doses of methacholine were added to the organ bath solution to obtain a rapid concentration-response curve (these data were not used). After a 30 min washing period (with six intermediate changes of buffer solution to remove methacholine), the first concentration-response curve with methacholine was performed (indicated as the control curve). Next, the tracheal rings were washed again and incubated with different concentrations of rhTNFα or buffer control for 30 min. Thereafter, the tracheal rings were subjected to a second concentration-response curve with methacholine, either directly (designated the direct curve) or after another 30 min washing period (designated the delayed curve). During the direct- or delayed-type experiments with TNFα, a buffer control curve was also obtained (designated blank curve).

In order to evaluate the dose-response effects, rhTNFα was administered to the tracheal strips in concentrations ranging 10⁻¹²–10⁻⁸ M. Dose-response effects were assessed in the delayed-type models (i.e. 30 min of washing after incubation with rhTNFα). Thereafter, in order to assess the specificity of the effect induced by rhTNFα, tracheal rings were simultaneously incubated with 10⁻¹³ M rhTNFα and 10⁻¹⁰ M rh-soluble TNF-receptor-p80. This concentration of rhTNF-receptor-p80 was shown previously to inactivate the bioactivity of TNFα completely, as assessed by the WEHI assay [15]. All experiments were performed pairwise in tissues from the same animals.

Since TNFα has been reported to enhance the activity of phospholipase-A₂ (PL-A₂) [18] with subsequent synthesis of prostaglandins and PAF; in the next set of experiments, tracheal strips were preincubated for 10 min with 10⁻⁴ M indomethacin or 10⁻⁵ M WEB 2086, followed by 30 min of incubation with 10⁻¹¹ M rhTNFα. These concentrations of indomethacin and WEB 2086 were considered to block endogenous prostaglandin and PAF effects completely [19]. The second concentration-response curve for methacholine was also performed after washing for 30 min, during which indomethacin and WEB 2086 were continuously present in the washing solution. As before, experiments were performed pairwise in tissues from the same animals.

Statistical analysis

Data are presented as mean±standard error of the mean (SEM) of at least eight independent experiments. For methacholine concentration-response curves, the contraction obtained following incubation with buffer control or TNFα was expressed as a percentage of the initial response (control curve). The results were statistically evaluated using Student’s t-test for paired data (two-sided test). A p-value <0.05 was considered statistically significant.

Results

Effect of TNFα on methacholine induced bronchoconstriction

Incubation of tracheal smooth muscle with 3×10⁻¹¹ M rhTNFα significantly increased the maximum response to methacholine (fig. 1). This enhanced response was, however, only observed in the delayed model (169.6±4.3%, p<0.005 in comparison with the control, blank and direct model). The blank curves (second methacholine concentration-response curve with buffer control) were not statistically different from the first concentration-response curves, indicating that intermediate changes of buffer or prolonged
presence in the organ bath did not influence the contractility of the tracheal strips within the duration of our experiments. During incubation with TNFα, the baseline tone of tracheal strips did not change. The -log concentration of drug needed to obtain half maximum response (pD2) values of the different curves, both with and without rhTNFα, were not statistically different from those of the control curves (table 1).

In the second set of experiments, the dose-response effects of different concentrations of rhTNFα (10−10–10−10 M) on methacholine-induced contraction of tracheal smooth muscle were evaluated. Considering the results above, in this and the next set of experiments, only the delayed model was used. Our experiments demonstrate that for all concentrations used, rhTNFα increased the maximum response to a similar extent (fig. 2).

**Experiments with selective inhibition of TNFα with soluble TNF-receptor construct**

In order to ascertain the specificity of action of rhTNFα on tracheal smooth muscle, in the next set of experiments, simultaneous incubation of 10−11 M rhTNFα and 10−10 M recombinant soluble TNFR-p80:Fc construct was used (fig. 3). rTNFR-p80:Fc was shown to inhibit the effect of TNFα on tracheal hyperreactivity completely.

**Experiments evaluating involvement of prostaglandins and PAF in TNFα-induced bronchial hyperresponsiveness**

Since TNFα increases PL-A₂-activity, tracheal rings were incubated with indomethacin to block endogenous prostaglandin release and with WEB 2086 to inhibit PAF effects. As observed before, incubation of tracheal rings with indomethacin alone did induce an increase in contractility, probably induced by shifts occurring within prostaglandin synthesis. Coincubation of tracheal rings with indomethacin (10−5 M) and rhTNFα did not influence induction of bronchial hyperreactivity by rhTNFα (fig. 4a), indicating that prostaglandin synthesis does not play a direct role in TNFα-induced hyperreactivity.

Table 1. pD2 values of concentration-response curves to methacholine of tracheal smooth muscle of the guinea-pig

<table>
<thead>
<tr>
<th>Concentration of rhTNFα (M)</th>
<th>pD2 Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.39±0.05</td>
</tr>
<tr>
<td>Blank</td>
<td>6.41±0.03</td>
</tr>
<tr>
<td>TNFα 10−10 M</td>
<td>6.71±0.25</td>
</tr>
<tr>
<td>TNFα 10−12 M</td>
<td>6.30±0.07</td>
</tr>
<tr>
<td>TNFα 10−11 M</td>
<td>6.50±0.16</td>
</tr>
<tr>
<td>TNFα 10−10 M</td>
<td>6.26±0.11</td>
</tr>
<tr>
<td>+TNFR-p80:Fc 10−10 M</td>
<td>6.37±0.16</td>
</tr>
<tr>
<td>TNFα 10−11 M</td>
<td>6.49±0.22</td>
</tr>
<tr>
<td>+WEB2086 10−10 M</td>
<td>6.34±0.04</td>
</tr>
</tbody>
</table>

pD2 values (-log concentration of drug needed to obtain half maximum response) of tracheal smooth muscle response to methacholine did not change significantly in all experiments performed. TNFα: tumour necrosis factor-α; TNFR-p80:Fc: tumour necrosis factor receptor (p80) linked to the Fc portion of human immunoglobulin (Ig)G1.

![Fig. 2.](image)

**Fig. 2.** Maximal isotonic contraction to methacholine of tracheal rings of the guinea-pigs without and with different concentrations of recombinant human tumour necrosis factor-α (rhTNFα). The curves were obtained after a 30 min washing period after incubation with tumour necrosis factor-α (TNFα) (30 min) and are the means±SEM of at least eight independent experiments. Con: control; B: blank. #: p<0.02 for all concentrations of rhTNFα versus the control and blank.

![Fig. 3.](image)

**Fig. 3.** Specific tumour necrosis factor-α (TNFα) inhibitor abrogates TNFα-induced bronchial hyperresponsiveness. Concentration-response curves (mean±SEM) of methacholine chloride on (at least eight) tracheal strips of the guinea-pigs without and with different concentrations of recombinant human tumour necrosis factor-α (rhTNFα) and 10−10 M rTNF-receptor-p80:Fc construct (▲). Curves were obtained 30 min after incubation with TNFα or buffer control. Experiments were performed pairwise. **: p<0.01 rhTNFα versus blank and simultaneous incubation of rhTNFα and rTNF-receptor-p80:Fc.

Treatment with WEB 2086 (10−6 M) alone did not influence reactivity of tracheal strips, whereas coinubcation of WEB 2086 with TNFα completely abrogated TNFα-induced hyperreactivity of tracheal smooth muscle (fig. 4b), demonstrating an involvement of PAF in the induction of tracheal hyperreactivity.

**Discussion**

The present study demonstrates that rhTNFα induces bronchial hyperreactivity in isolated tracheal rings of the guinea-pig. The specificity of the effect was ascertained by a specific TNFα-antagonist that completely inhibited the induction of bronchial hyperreactivity. Additionally, we demonstrated that PAF is involved in the induction of hyperreactivity by TNFα.
TNFα has been shown to be involved in the development of BHR, both in animal models [4] as well as in humans [10]. These studies did not show whether TNFα affects bronchial smooth muscle directly or whether TNFα initiates mediator release from other cells within the pulmonary compartment, which secondarily induce BHR. In the present study, rhTNFα induced BHR in isolated tracheal rings of the guinea-pig. The effects of TNFα were observed at concentrations comparable with the levels found in BAL fluid of asthmatic patients (10^{-12} M) [5].

So far, few studies have evaluated the effects of TNFα on bronchial contractility in vitro. The limited data available are difficult to interpret due to different experimental designs. Prolonged incubation (18 h) with TNFα (100 ng·mL^{-1}) of isolated tracheas of sensitized guinea-pigs did not affect carbachol-induced maximum contraction [20]. More recently, the effect of rhTNFα on isometric contractility of human bronchial rings was evaluated [21]. In this study, TNFα increased the maximum response to 113%. In accordance with the present study, the investigators also observed a time delay before the onset of the effect, with the first response starting between 8 and 16 min after incubation. Concentrations above 10^{-6} M rhTNFα induced an increased response, and in agreement with our observations, no clear dose-response effect with regard to TNFα-induced hypercontractility was observed.

TNFα has been shown to enhance PL-A2 activity in various cells, leading to synthesis of PAF [18, 22]. Our experiments demonstrated that PAF is involved in the TNFα-induced hypercontractility of tracheal rings in vitro. PAF has a wide range of target cells, including smooth muscle cells. Moreover, PAF has been shown to induce BHR to histamine and methacholine in guinea-pigs [23, 24]. This study did not clarify the cellular source of PAF synthesis in tracheal rings after stimulation with TNFα. TNFα has been shown to induce PAF synthesis within cells present in tracheal rings, such as endothelial and bronchial epithelial cells and also in macrophages, monocytes and neutrophils [22, 25, 26]. Studies with activated rat macrophages have shown that after TNFα-activation, at least 1 h is needed to detect PAF in supernatant, whereas endothelial cells release PAF not before 2 h [22]. In the previous experiments, PAF was shown to be largely retained within the cell in the first few hours following stimulation. Considering the time delay in our experiment after TNFα stimulation, the present study suggests that either smooth muscle cells in tracheal rings interact with cells expressing PAF at their surface or synthesize PAF themselves [27]. The present experiment does not permit us to specify the cellular source of PAF synthesis.

TNFα was also shown to influence intracellular [Ca^{2+}] in smooth muscle cells of guinea-pigs [28]. TNFα (10 ng·mL^{-1}) induced large increases in cytosolic free [Ca^{2+}] after bradykinin stimulation. Since PAF synthesis is associated with calcium mobilization, PAF synthesis may also be involved in these experiments.

The present study did observe a steep dose-response effect for TNFα-induced hypercontractility. This observation concurs with studies in human bronchial rings [21]. A steep dose-response effect for TNFα-induced PAF synthesis was observed in macrophages and endothelial cells [22], whereas monocytes did not demonstrate a dose-response effect [26]. In these experiments, TNFα concentrations as low as 1 ng·mL^{-1} were shown to induce PAF synthesis, whereas PAF has been shown to possess a median effective concentration (EC50) value of 10 pM in isolated ileal smooth muscle cells of guinea-pigs [29]. Therefore, the absence of a clear dose-response effect of TNFα in the present study may be explained by assuming a threshold for TNFα effects.

In humans, both TNFα and PAF have been implicated in the inflammatory process of asthma [1, 6]. So far, studies with PAF antagonists in asthma have yielded disappointing results. However, recently, a new, very efficient PAF antagonist was shown to reduce BHR in stable asthmatic patients [30]. The importance of PAF in asthma was further emphasized by the demonstration of a deficiency in PAF-acetylhydrolase activity in a subgroup of asthmatic patients [31]. If TNFα in vivo leads to PAF production,
selective inhibition of TNFα itself or its release may prove more efficient in asthma treatment than PAF antagonists. In this context, it should be mentioned that a potent in-hibitory TNF-receptor construct was recently shown to reduce both BHR and inflammatory cell influx in the late asthmatic response in guinea-pigs [32].

In conclusion, our study shows that tumour necrosis factor-α induces hyperreactivity in tracheal rings and that this hyperreactivity is platelet activating factor-mediated. Tumour necrosis factor-α represents a common pathway for different stimuli to generate bronchial hyperresponsiveness in respiratory disease, like allergic inflammatory lung disease, viral and bacterial infections and in occupational asthma. Considering the central and pro-inflammatory role of tumour necrosis factor-α in the pathogenesis of asthma, early inhibition of events leading to bronchial hyperresponsiveness using specific (inhaled) tumour necrosis factor-α-antagonists or tumour necrosis factor-α-release blocking agents may prove beneficial.

References


