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Short communication

Induction of human airway hyperresponsiveness by tumour necrosis factor-α

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Abstract

Tumour necrosis factor-α (TNFα) is implicated in the pathogenesis of asthma; however, little is known of its direct effect on smooth muscle reactivity. We investigated the effect of TNFα on the responsiveness of human bronchial tissue to electrical field stimulation in vitro. Incubation of non-sensitized tissue with 1 nM, 3 nM and 10 nM TNFα significantly increased responsiveness to electrical field stimulation (113 ± 8, 110 ± 4 and $112 \pm 2\%$ respectively) compared to control ($99 \pm 2\%$) ($P < 0.05$, $n = 6$). Responses were not increased in sensitized tissue ($101 \pm 3\%$ versus $105 \pm 5\%$, $n = 3$, $P > 0.05$) nor were responses to exogenous acetylcholine ($93 \pm 4\%$ versus $73 \pm 7\%$, $n = 3$, $P = 0.38$). These results show that TNFα causes an increase in responsiveness of human bronchial tissue and that this occurs prejunctionally on the parasympathetic nerve pathway. This is the first report of a cytokine increasing human airway tissue responsiveness.

Keywords: Asthma; TNF-α (tumor necrosis factor-α); Cytokine; Bronchial hyperresponsiveness; Electrical field stimulation; Airway, human

1. Introduction

Asthma is a chronic disease of the airways characterised by and associated with many inflammatory products, including cytokines. Many cytokines or their mRNA have been detected in increased quantity in bronchial biopsies or bronchoalveolar lavage fluid from asthmatic subjects. Tumour necrosis factor-α (TNFα), granulocyte-macrophage colony stimulating factor and interleukins 1–6 have been detected in human airways (Broide et al., 1992; Robinson et al., 1992).

The multi-functional pro-inflammatory cytokine, TNFα, may be of importance in asthma as it is known that TNFα is increased in the sputa of patients with bronchial asthma (Taki et al., 1991). TNFα is also present in the bronchoalveolar lavage fluid of patients with symptomatic asthma (Broide et al., 1992).

The role of TNFα in asthma is not fully understood. Previously, we have shown that TNFα increases the release of histamine from human lung mast cells (Hughes et al., 1994). Studies in animals have demonstrated that TNFα induces airway hyperresponsiveness to aerosolised histamine in vivo (Wheeler et al., 1990; Kips et al., 1992) and inhaled TNFα increases the responsiveness of normal subjects to methacholine (Yates et al., 1993). Whether this is a direct effect on the contractile responsiveness of the airway muscle or due to an indirect mechanism is not known.

In our previous studies we have investigated the effect of sensitization on human airway tissue responsiveness. Responsiveness to certain agonists is greatly increased in tissue which is sensitized (Black et al., 1990).

The purpose of this study was to determine whether TNFα alters the responsiveness of human bronchial tissue in vitro and if so, to determine the site of action. The effect of TNFα on tissue responsiveness was studied in sensitized and non-sensitized specimens. The effect of TNFα on human airway responsiveness was studied on isolated bronchial tissue stimulated with electrical field stimulation, while the site of action of TNFα was examined using submaximal exogenous doses of acetylcholine.

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2. Materials and methods

2.1. Response to electrical field stimulation

Samples of human lung tissue were obtained from specimens surgically removed either at thoracotomy from patients with pulmonary carcinoma or from patients with emphysema undergoing pulmonary transplantation. Approval for all experiments with human lung was provided from the Human Ethics Committee of the University of Sydney. The patients ranged in age from 54 to 68 years (mean ± standard deviation, 60.5 ± 5.7). The lung samples were placed in Krebs-Henseleit solution which had previously been aerated with carbogen (5% carbon dioxide in 95% oxygen) and were transported to the laboratory on ice. Whenever possible, the experiment was conducted on the same day as the removal of the specimen. However, on some occasions the tissue was placed in freshly aerated Krebs-Henseleit solution, and stored at 4°C overnight. Bronchi were dissected free from surrounding parenchyma and blood vessels and cut into rings 3–5 mm in length. The bronchial rings were mounted on Perspex rods equipped with stainless steel electrodes which were connected to Grass SD9 stimulators. The tissue was equilibrated against a 1–2 g load as previously described (Armour et al., 1988) in 5 ml siliconized water-jacketed organ baths containing Krebs-Henseleit solution at 37°C and constantly aerated with carbogen. The tissue was allowed to equilibrate over a 1 h time period during which time the Krebs-Henseleit solution was changed every 15 min. Changes in tension were measured isometrically with Grass FTO3 tranducers and recorded on a Grass polygraph.

When a stable baseline was attained, the sensitization status of the tissue was determined using one tissue. Sensitization status was determined using three common allergens (*Dermatophagoides pteronyssinus*, *Alternaria tenuis* and *Phleum pratense*). 10 μl of each of the allergen extracts were added to the organ bath one at a time, allowing 20 min between additions. The tissue was classified as sensitized if it contracted to one or more of the three allergens. If the tissue did not contract to any of the three allergens and it contracted to carbachol (10^{-3} M) it was considered to be non-sensitized. The tissue(s) used to test sensitization status were not used again during the course of the experiment.

Electrical field stimulation was applied to the remaining tissues. These tissues were stimulated at 1 ms for 20 s every 4 min by an automated timing device using a Grass SD9 stimulator. The frequency and voltage used ranged from 10 to 70 V and 4 to 16 Hz and depended on the magnitude of the tissue response. The voltage and frequency were adjusted in order to attain responses between 200 and 600 mg increase in tension. Each tissue was then stimulated with a constant voltage and frequency until three responses within 10% of one another were obtained. At this stage, the response of the tissue was considered to be stable and 50 μl of either vehicle (control), or one of five dilutions of TNFα was added to separate tissues to give final TNFα concentrations of 0.01 nM, 0.1 nM, 1 nM, 3 nM and 10 nM. The electrical field stimulation was repeated 2 min later and then every 4 min. The subsequent response to electrical field stimulation was observed for a total of 20 min. At this stage, 10^{-6} M atropine was added and the response observed until completely inhibited (3–6 stimulations). Thus parasympathetic nerve stimulation was confirmed.

2.2. Site of action

In a separate series of experiments, the effect of TNFα on the response of the tissue to exogenous acetylcholine was investigated. After the tissue had equilibrated to the applied load (as above), a single appropriate submaximal dose of acetylcholine, ranging from 10^{-7} M to 3×10^{-5} M was added to the tissue. The appropriate concentration of acetylcholine for each tissue was the concentration that produced a contraction equivalent to between 200 and 600 mg of tension. The contractile response was allowed to plateau, the response measured in mg of tension, the tissue washed and allowed to return to baseline tension. This procedure was repeated until three consecutive responses within 10% of one another were obtained. At this stage 50 μl of vehicle was added to one tissue (control) while 50 μl of TNFα (giving a final TNFα concentration of 1 nM) was added to another tissue, followed 2 min later by the appropriate concentration of acetylcholine. The tissue was then washed and allowed to return to baseline for 30 min during which time it was washed every 15 min. When a stable baseline was attained a maximal dose of carbachol (10^{-3} M in organ bath) was added to each tissue.

2.3. Materials

Acetylcholine and carbamylcholine chloride (carbachol) were obtained from Sigma. Atropine was purchased from Astra Pharmaceuticals. *Dermatophagoides pteronyssinus*, *Alternaria tenuis* and *Phleum pratense* extracts for scratch test were purchased from Hollister-Stier (Division of Miles, Indiana, USA). Human recombinant TNFα (> 97% pure by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and N-terminal sequence analysis) was obtained from Pierce as a powder which was reconstituted in distilled water on receipt (0.1 mg/ml). LAL assay (*Limulus* amebocyte lysate assay) shows no detectable endotoxin contamination of the TNFα sample

at the sensitivity level of the assay (< 0.06 Eu/ml). Validation tests show no product inhibition or enhancement by the TNFα sample. The vehicle (control) for TNFα was distilled water. Aliquots of the preparation were stored at −20°C to avoid repeated freeze-thawing. The TNFα was thawed and diluted appropriately in distilled water immediately before use.

2.4. Analysis of results

For the electrical field stimulation experiments, the mean of the three stable responses obtained prior to addition of vehicle, or the three dilutions of TNFα was designated as the 'initial response' and the responses following the addition of vehicle or TNFα were each expressed as a percentage of the 'initial response'. The maximum response (%) obtained in the 20 min following addition of vehicle or TNFα was recorded for statistical analysis. A mean value was obtained for the maximum responses for each treatment from nine separate experiments. The mean maximum percentage responses upon addition of vehicle and the five concentrations of TNFα (0.01 nM, 0.1 nM, 1 nM, 3 nM and 10 nM) were compared using analysis of variance and the Fisher test.

For the acetylcholine experiments, the contraction obtained following the addition of vehicle or TNFα was expressed as a percentage of the 'initial contraction' where the 'initial contraction' was the mean of the three reproducible contractions obtained prior to their addition. A mean value was obtained from the responses (%) in each experiment following the addition of vehicle or TNFα and compared using a two-tailed paired Student's *t*-test. Results were considered significant when $P < 0.05$.

3. Results

3.1. Non-sensensitized tissue

A significant increase in responsiveness of human bronchial tissue to electrical field stimulation was observed in the presence of 1 nM, 3 nM and 10 nM TNFα when compared to the control. The responsiveness of the human bronchial tissue to electrical field stimulation in the presence of 1 nM, 3 nM and 10 nM TNFα was not significantly different from one another. The response of the tissue was 113 ± 8%, 110 ± 3% and 112 ± 1% in the presence of 1 nM, 3 nM and 10 nM TNFα respectively and 99 ± 3% in the control ($n = 9$, $P < 0.05$). The increase in responsiveness developed within the first 20 min and usually between 8 and 16 min. The maximum potentiation most commonly occurred at 12 min, following the addition of TNFα. The magnitude of the potentiation began to decrease following the attainment of maximum potentiation. The lower concentrations of TNFα, 0.01 nM and 0.1 nM, caused slight increases in bronchial tissue responsiveness (107 ± 13% and 103 ± 10% respectively) but these were not significantly different from control (Fig. 1).

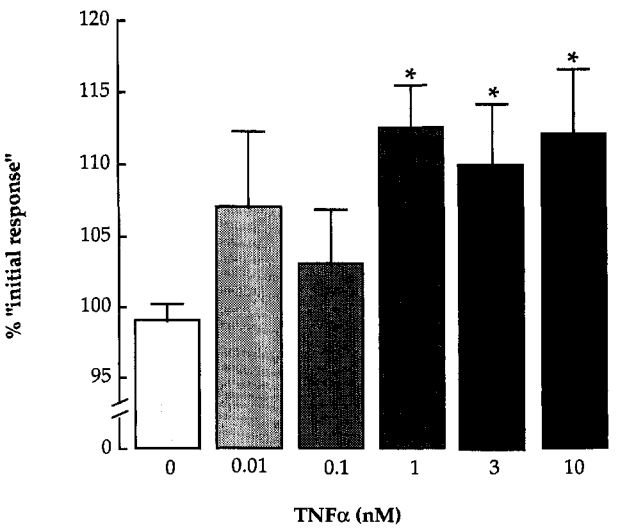

Fig. 1. Responsiveness of human bronchial tissue following incubation with vehicle (0), 0.01, 0.1, 1, 3 and 10 nM TNFα expressed as a percentage of 'initial response'. 'Initial response' is the mean of the three stable responses obtained prior to the addition of either vehicle or TNFα. Vertical bars denote the S.E.M. values. *Significant difference from control (vehicle), $n = 9$, $P < 0.05$, ANOVA, Fisher test.

1 nM TNFα, which caused an increase in responsiveness of human bronchial tissue to electrical field stimulation, did not significantly increase responsiveness to exogenous acetylcholine (Fig. 2).

3.2. Sensitized tissue

In sensitized tissues responses in the presence of 0.01, 0.1 and 1 nM TNFα were 104 ± 1%, 100 ± 2% and 105 ± 5% respectively ($n = 3$). These responses were not significantly different from control (101 ± 3%) (data not shown). Thus the effect of TNFα on the acetylcholine responsivenes was not tested.

4. Discussion

Our results demonstrate that 1 nM, 3 nM and 10 nM TNFα cause an increase in the response of non-sensitized human bronchial tissue to electrical field stimulation in vitro. This is not a dose-dependent effect. Our results further show that TNFα acts prejunctionally at the site of the parasympathetic nerve innervating the bronchial tissue. TNFα had no effect on the response of sensitized human bronchial tissue to electrical field stimulation.

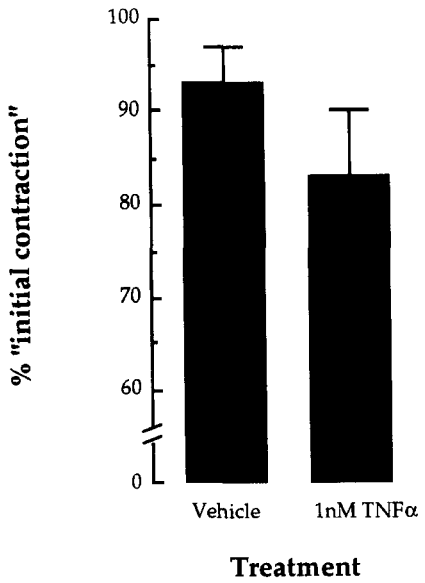

Fig. 2. Responsiveness of human bronchial tissue to acetylcholine following incubation with vehicle or 1 nM TNFα expressed as a percentage of 'initial contraction', where 'initial contraction' is the mean of the three stable contractions obtained prior to addition of either vehicle or TNFα, $n = 3$. Vertical bars denote the S.E.M. values.

In order to determine the effect of TNFα on human bronchial responsiveness five specific concentrations of TNFα were tested (0.01, 0.1, 1, 3, 10 nM). The concentrations of TNFα used in the experiments reported here were calculated based on published data (Dubravec et al., 1990). Thus neutrophils (5×10^5) upon stimulation released TNFα in the concentration of 160–200 pg/ml (9.41×10^{-12}–1.18×10^{-11} M). In previous experiments, in which we have demonstrated that neutrophil products increased responsiveness of human bronchial tissue to electrical field stimulation (unpublished data), we used more neutrophils (5×10^6). Under these circumstances more TNFα would be released than that described by Dubravec et al. (1990).

In an attempt to study the effect of TNFα on the responsiveness of human bronchial tissue, electrical field stimulation was chosen as a stimulus. Electrical field stimulation is an established method for stimulating human airways in vitro and the contraction observed is due to release of acetylcholine from parasympathetic nerves (Black et al., 1990). We confirmed this in the present study as atropine inhibited the electrical field stimulation response we observed.

Having established that TNFα increases the responsiveness of non-sensitized human bronchial tissue to electrical field stimulation it was of interest to determine the site of action of TNFα. The site of action was prejunctional since TNFα did not increase the response of the muscle to exogenous acetylcholine which would indicate a postjunctional location.

In contrast to the effect observed in non-sensitized tissue, TNFα had no effect on the responsiveness of sensitized tissue to electrical field stimulation. It is possible that sensitized airways have been subjected to infiltration by inflammatory cells many of which are a source of TNFα. The previous exposure of the tissue to TNFα could lead to desensitization and thus any exogenous TNFα added might not produce an effect.

TNFα has been shown to be involved in the inflammatory process in asthma. In the lung, neutrophils, mast cells, alveolar macrophages, airway epithelial cells and T-lymphocytes are all potential sources of TNFα (Dubravec et al., 1990; Gosset et al., 1991; Ohkawara et al., 1991; Rochester and Rankin, 1991; Gordon and Galli, 1991). Given that these cells are either present in the airways or infiltrate the airways in asthma, one or all of these cells may contribute to the increased level of TNFα found in the sputa of asthmatics (Taki et al., 1991) and the increased level of TNFα in the bronchoalveolar lavage fluid of patients with symptomatic asthma (Broide et al., 1992). We have previously shown that products of activated inflammatory cells increase human bronchial responsiveness to electrical field stimulation in vitro (Hallahan et al., 1990). This study shows that the inflammatory cell product TNFα is capable of inducing similar hyperresponsiveness of human bronchial tissues in vitro and thus TNFα has the potential to increase responsiveness in human airways in asthma. This is the first report of a cytokine potentiating responses in human tissue. The present findings complement our previous observations, which showed that TNFα increases the release of histamine from human lung mast cells (Hughes et al., 1994) and demonstrate that TNFα has the potential to be actively involved in the pathogenesis of asthma. When combined with the observations of others that TNFα induces in vivo hyperresponsiveness in both animals (Wheeler et al., 1990; Kips et al., 1992) and humans (Yates et al., 1993) we can conclude that the proinflammatory cytokine TNFα is likely to play a significant role in asthma.

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