

Vitamin E down-modulates mitogen-activated protein kinases, nuclear factor- κ B and inflammatory responses in lung epithelial cells

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Introduction

The lung epithelium presents the first physical barrier to inhaled oxidants. In addition to direct oxidant–epithelial interactions, proinflammatory mediators secreted from other pulmonary cells in response to the oxidant challenge can also act upon epithelial cells indirectly. One such mediator is tumour necrosis factor (TNF)- α , which activates intracellular signal transduction pathways in bronchial epithelial cells resulting in increased cell surface expression of adhesion molecules implicated in the recruitment of leucocytes to inflammatory loci [1]. In addition, TNF- α can induce epithelial cells to secrete other cytokines such as interleukin (IL)-8, an important neutrophil chemoattractant [2].

The lung injury caused by oxidative stress and excessive inflammatory responses is counteracted by naturally occurring anti-oxidants such as vitamin E (tocopherol). Plasma

Summary

The airway epithelium plays an active role in acute lung inflammation by producing chemotactic factors and by expressing cell adhesion molecules involved in the migration of leucocytes to extravascular spaces. We have reported previously that neutrophil migration to airways can be down-modulated by exogenously administered vitamin E (α -tocopherol). The mechanism for this effect is not well understood, however. The action of α -tocopherol was investigated in human alveolar type II and bronchial epithelial cells stimulated with tumour necrosis factor- α . Treatment of alveolar epithelial cells with α -tocopherol resulted in down-regulated cell surface expression of intercellular adhesion molecule-1 (ICAM-1). On bronchial epithelial cells, both ICAM-1 and vascular adhesion molecule-1 were decreased, leading to diminished adherence of leucocytes to the cells. The production of the neutrophil chemoattractant interleukin-8 was attenuated in both alveolar and bronchial cells. These effects were preceded by reduced activation of the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK1/2) and p38, as well as down-regulation of nuclear factor- κ B. Comparing the effects of α -tocopherol with that of specific inhibitors of MAPK and protein kinase C (PKC) revealed that effects appear to be partly independent of PKC inhibition. These results implicate the anti-inflammatory action of α -tocopherol in addition to its anti-oxidant properties.

Keywords: ARDS, asthma intervention, lung epithelial cells, MAPkinase, NF- κ B, vitamin E

levels of α -tocopherol, a member of the vitamin E family, are decreased after infections, trauma, burns and inflammatory injuries, indicating that this anti-oxidant is depleted during acute tissue injury [3]. Kelly *et al.* have demonstrated that lung lining fluid α -tocopherol concentrations are low in patients with asthma due to ongoing airway inflammation, and are thus potentially more sensitive to additional oxidative insults [4]. Consistent with this, dietary supplementation of asthmatics with vitamin E has been shown to protect against the effects of oxidant air pollutants such as ozone [5,6]. Further, higher vitamin E intake is associated with lower serum IgE and a decreased frequency of allergen sensitization [7]. Administration of exogenous α -tocopherol incorporated in liposomes protects against lung oedema in animal models of acute respiratory distress syndrome (ARDS) provoked by systemic inflammation [8–10] and chemical-induced lung injury

caused by inhaled bleomycin [11]. We have demonstrated that systemically administered α -tocopherol reduces transendothelial migration of neutrophils and prevents lung injury in a mouse model of lipopolysaccharide (LPS)-induced airway inflammation [12], and improves respiratory function in a mouse model of septic shock induced by two consecutive intraperitoneal injections of LPS (Shwartzman reaction) [13]. These results are consistent with studies *in vitro* showing that α -tocopherol inhibits polymorphonuclear (PMN) leucocyte-dependent adhesion to the endothelium by suppressing the expression of cell adhesion molecules such as CD11b/CD18 on neutrophils, and/or intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells [14,15].

The pulmonary uptake of α -tocopherol is augmented in acute lung injury [16], suggesting that systemic delivery of this anti-oxidant may target the lung. Kolleck and colleagues have suggested that lipoprotein bound to α -tocopherol was delivered to alveolar type II cells with uptake mediated by lipoprotein-specific receptors [17,18]. Lipid soluble vitamin E acts in cell membranes, where it prevents the propagation of lipid peroxidation reactions [19,20].

However, it is recognized that vitamin E, particularly α -tocopherol, exerts a number of non-anti-oxidant functions, some associated with inhibition of protein kinase C (PKC) [21] such as inhibition of platelet aggregation [22], and others independent of PKC such as the expression of ICAM-1 [23], integrins [24] and CD36 [25]. These later observations may be tracked back, at least in part, to the reported inhibition of the transcription factor nuclear factor (NF)- κ B [26]. Most previous studies of α -tocopherol actions have been performed on cells derived from circulation, while the intracellular effects of this drug in lung epithelial cells have been poorly investigated.

The aim of the present study was to investigate the effects of α -tocopherol on bronchial and alveolar epithelial cells stimulated with TNF- α . Both transformed cell lines and primary bronchial epithelial cells were used. We demonstrated that α -tocopherol down-regulates cell surface expression of ICAM-1 and secretion of interleukin (IL)-8, both in bronchial cells and cells of alveolar type II origin. Because the transcription factors NF- κ B and activator protein-1 (AP-1) are key regulatory molecules involved in such a response, it was decided to investigate if α -tocopherol affects the activation of these factors. The mechanisms for the inhibition were investigated further by analysis of upstream signal transduction pathways induced by TNF- α , i.e. phosphorylation of mitogen-activated protein kinases (MAPK). The inhibitory effects of α -tocopherol were also compared with those of MAPK, PKC and I κ B inhibitors. Our results indicate that α -tocopherol inhibits epithelial cell function by down-regulating activation of extracellular signal-regulated kinase (ERK), p38 and NF- κ B, effects that appear to be partly independent of PKC inhibition.

Materials and methods

Cell culture and treatments

A549 cells (ATCC CCL-185; American Type Culture Collection), a human type II alveolar epithelial cell line, were cultured in RPMI-1640 (GIBCO BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS; HyClone, Perbio Science, Aalst, Belgium) and 50 μ g/ml gentamicin. Bronchial epithelial cell line (BEAS)-2B, human bronchial epithelial cells transformed by an adenovirus 12 SV40 hybrid (ATCC CRL-9609) and normal human bronchial epithelial cells (NHBE) (Clonetics, San Diego, CA, USA) were grown in serum-free bronchial epithelial cell basal medium (BEBM) with supplements (complete medium, BEGM) (Cambrex, Verviers, Belgium). BEAS-2B and NHBE were cultured on tissue culture flasks or plates precoated with fibronectin, vitrogen and bovine serum albumin. U937, a human monocytic leukaemia cell line (ATCC CRL 1593), was cultured in RPMI-1640 medium supplemented with 10% FCS and 50 μ g/ml gentamicin. All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO₂. Vitamin E (α -tocopherol: Sigma, St Louis, MO, USA), was dissolved in dimethyl sulphoxide (DMSO) with a final concentration of solvent in the culture medium not exceeding 0.1%. Cells were exposed to various concentrations of α -tocopherol for 45 min in a concentration range of 25–100 μ M prior to stimulation with 10 ng/ml TNF- α (Sigma) for time-periods indicated in the figure legends. Cells exposed only for solvent served as controls. Treatment of cells with α -tocopherol alone had no effect on any of the parameters tested. In all experiments cell cultures with 80% confluence were used.

Analysis of IL-8 in cell supernatant by enzyme-linked immunosorbent assay (ELISA)

Lung epithelial cells were seeded in 24-well plates in complete growth medium. After 48 h, cells were stimulated with 10 ng/ml TNF- α and incubated for another 24 h. Some wells were preincubated with α -tocopherol 45 min before addition of TNF- α . In a set of experiments the MAP kinase (MEK) inhibitor PD98059 (Calbiochem, San Diego, CA, USA), the p38 inhibitor SB203580 (Calbiochem) or the PKC inhibitor Calphostin C (Sigma) were added 45 min before TNF- α stimulation. To study the effects of NF- κ B inhibition, the inhibitor of kappa B (I κ B) BAY 11-7082 (Calbiochem) was added 60 min before TNF- α activation. The optimal concentration of each inhibitor was determined in pilot experiments. PD98059 was used at 40 μ M, SB203580 at 1 μ M, Calphostin C at 0.5 μ M and BAY 11-7082 at 8 μ M in A549 cell cultures and at 1 μ M in BEAS-2B cell cultures. The supernatants were separated by centrifugation, and the concentration of IL-8 was measured using the DuoSet ELISA Development kit (R&D Systems, Abingdon, UK), according to the manufacturer's protocol.

Flow cytometry analysis

Cells were grown in 25 cm² tissue culture flasks and stimulated with 10 ng/ml of TNF- α (Sigma) for 20 h, or pretreated for 45 min with α -tocopherol in a concentration range of 25–100 μ M prior to addition of TNF- α . In some experiments the inhibitors PD98059, SB203580 or Calphostin C were added 45 min before TNF- α stimulation. In another set of experiments the I κ B inhibitor BAY 11–7082 was added 60 min before TNF- α activation. Optimal concentration of each inhibitor was determined in pilot experiments. The treated cells were rinsed with phosphate-buffered saline (PBS) and detached from tissue culture flasks using Versene or Accutase. The cells were washed by centrifugation and resuspended in 3% FCS–PBS followed by distribution of 1×10^6 cells into wells in a round-bottomed microtitre plate. Monoclonal phycoerythrin (PE)-conjugated anti-ICAM-1 (CD54; Pharmingen, San Diego, CA, USA), VCAM-1 (CD106; Pharmingen) or isotype-matched (IgG₁ κ ; Pharmingen) antibody was then added to the wells. After incubation for 45 min in the dark (4°C), the cells were washed twice with 3% FCS–PBS, fixed with Cellfix (Becton Dickinson, San Jose, CA, USA) and transferred to fluorescence activated cell sorter (FACS) tubes (Becton Dickinson). The level of surface expression of ICAM-1 and VCAM-1 on the lung epithelial cells was determined by analysing specific median fluorescence intensity in FL1 of 10 000 cells using a FACSsort flow cytometer (Becton Dickinson).

Cell adhesion assay

U937 cells (2×10^6) were labelled with 50 μ l 1 mCi/ml Cr⁵¹ (Amersham Biosciences, Uppsala, Sweden) for 1 h at 37°C and washed three times with cold RPMI-1640. Thereafter, 0.5×10^5 of the labelled cells were added to the treated lung epithelial cells in a final volume of 500 μ l and incubated in 37°C (5% CO₂) for 45 min. Non-adherent cells were removed by gentle washing with RPMI-1640 four times. Thereafter, 300 μ l 5% Triton was added to lyse the cells. After 20 min incubation at room temperature the lysates were harvested and the radioactivity in the wells was estimated in a gamma-counter. As positive controls for the inhibition of cell adhesion, saturating amounts of anti-ICAM-1 and anti-VCAM-1 monoclonal antibody (MoAb) (Genzyme Corporation, Cambridge, MA, USA) were added and incubated for 30 min at 37°C prior to addition of U937.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extract were prepared from 2×10^7 cells. Treated cells were washed with ice-cold PBS and harvested using a cell scrape. Thereafter, cells were suspended in 5 ml ice-cold PBS and centrifuged at 250 g for 10 min at 4°C. The pellet

was dissolved in 100 μ l hypotonic buffer [10 mM HEPES, pH 7.3, 10 mM KCl, 1.5 mM MgCl₂, 2 mM Pefablock SC; Roche, Basel, Switzerland, 1 mM dithiothreitol (DTT), 0.2 mM ethylenediamine tetraacetic acid (EDTA) and complete protease inhibitors; Roche] and centrifuged at 5000 g for 1 min at 4°C. The cells were then resuspended in lysis buffer (hypotonic buffer containing 0.4% NP-40) and incubated for 10 min on ice followed by centrifugation at 8000 g for 1 min at 4°C. The nuclei pellets were mixed rapidly in 0.3 M KCl buffer (20 mM HEPES pH 7.3, 22% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.3 M KCl, 1 mM DTT and complete protease inhibitors) and left on ice for 30 min. After centrifugation at 8000 g for 15 min at 4°C, aliquots of supernatant containing the nuclear proteins were collected and stored at –80°C. The nuclear protein content of the supernatant was determined by Coomassie blue reaction using a Bio-Rad kit (Richmond, CA, USA). For detection of NF- κ B and AP-1 DNA binding activities in nuclear extracts, electrophoretic mobility shift assays (EMSA) were conducted as described previously [12]. For specificity analysis, a molar excess of unlabelled probe was added to the binding reaction. The sequences of oligonucleotides used to detect the DNA-binding activity were as follows; NF- κ B: 5'-AGTTGAGGGGACTTCCAGGC-3' and AP-1: 5'-CGCTTGATGAGTCAGCCGAA-3' (only the upper strands are indicated). The oligonucleotides were end-labelled using γ -[³²P] ATP (3000 Ci/mmol; NENLife Science Products, Boston, MA, USA) and T4 polynucleotide kinase (Promega, Madison, WI, USA). The labelled probes were purified from free nucleotides on a microspin G-50 M column (Amersham Biosciences).

Western blot analysis

A549 cultures were incubated in serum-free medium and bronchial epithelial cell line (BEAS)-2B cultures in basal BEBM medium overnight before treatment with or without α -tocopherol and exposure of TNF- α for 15 min. Cells were harvested in ice-cold PBS and centrifuged at 250 g at 4°C for 10 min.

Lysates were prepared by adding 200 μ l of 1 \times sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) to the cell pellets, and the samples were then sonicated for 15 s to shear DNA. Twenty-five μ l of cell lysates were loaded onto 12% precast SDS–polyacrylamide gel electrophoresis (PAGE) gels (Cambrex Bio Science, Rockland, ME, USA) and electrophoresed at 100 V for 45 min. The proteins were then electrotransferred to Immobilon-P Transfer membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% w/v non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS), immunoblotted with rabbit anti-phospho-ERK1/2 or anti-phospho-p38 antibodies (Cell Signalling Technology, Beverly, MA, USA) overnight at 4°C, followed by incubation with a secondary

horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signalling Technology) for 1 h. Membranes were washed extensively with TTBS after each incubation. Detection was performed using enhanced chemiluminescence (ECL; Amersham Biosciences) followed by autoradiography. To determine equal loading of cell lysate proteins, the membranes were stripped with a buffer containing 62.5 mM pH 6.8 Tris-HCl, 2% SDS and 100 mM β -mercaptoethanol for 30 min at 52°C and reprobed with anti-total ERK1/2 or anti-total p38 antibodies (Cell Signalling Technology).

Statistical analyses

Mean values and standard deviations were calculated and shown in the figures. The tests used for analyses of statistical significance are indicated in figure legends. $P < 0.05$ (two-tailed) was regarded as significant ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$). Calculations were performed using the computer programme SPSS (SPSS Inc., Chicago, IL, USA).

Results

α -Tocopherol down-modulates TNF- α -induced production of IL-8

The ability of α -tocopherol to inhibit the recruitment of neutrophils by down-modulating chemotactic activity was studied *in vitro*. As IL-8 is considered to be an important chemotactic factor for neutrophils we investigated if α -tocopherol affects the production of this cytokine in lung epithelial cells. In both bronchial (BEAS-2B and NHBE) and alveolar (A549) cells, the secretion of IL-8 was increased after 24 h incubation with TNF- α (Fig. 1). The highest concentration of IL-8 was achieved in supernatants from A549 cell cultures. In A549 and BEAS-2B cells the most efficient inhibition of IL-8 was observed with 50 μ M α -tocopherol. At this concentration, the production was reduced to 46%, in A549 cells to 45% in NHBE cells and to 33% in BEAS-2B cells of TNF- α -induced production. α -tocopherol completely attenuated the effect of TNF- α in NHBE cells and nearly reduced the IL-8 secretion to baseline level in BEAS-2B cells.

α -Tocopherol reduces TNF- α -induced cell surface expression of ICAM-1 and VCAM-1

The effect of α -tocopherol on TNF- α -induced expression of adhesion molecules ICAM-1 and VCAM-1 on lung epithelial cells was analysed by flow cytometry. The median fluorescence intensity of the anti-ICAM-1 antibody on unstimulated BEAS-2B and NHBE cells was approximately three times above the fluorescence intensity of the isotype-matched IgG control antibody, indicating a significant constitutive expression of ICAM-1 on the bronchial cells. On the other hand, baseline expression of ICAM-1 on the alveolar A549 cell line was undetectable as the median fluorescence

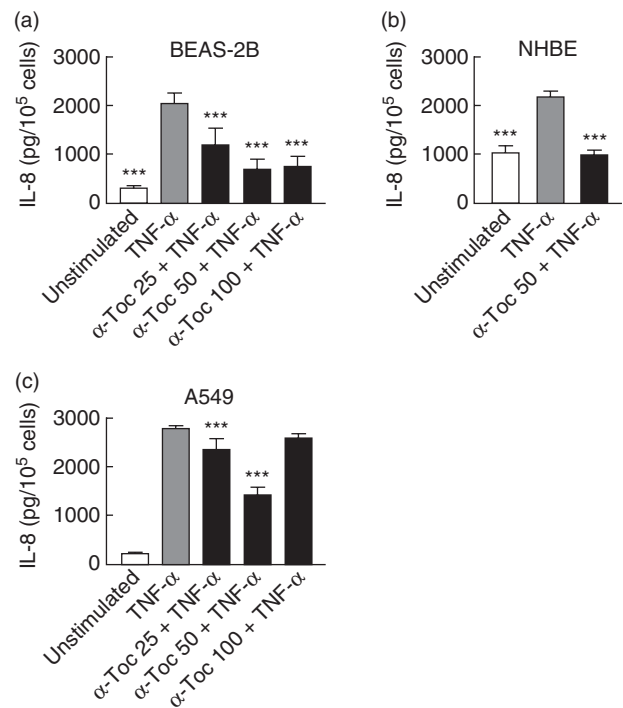


Fig. 1. Effect of α -tocopherol on interleukin (IL)-8 secretion from lung epithelial bronchial epithelial cell line (BEAS-2B) (a), normal human bronchial epithelial cells (NHBE) (b) and A549 (c) cells. Cells were treated for 45 min with α -tocopherol in a concentration range of 25–100 μ M and stimulated thereafter with 10 ng/ml tumour necrosis factor (TNF)- α . After 24-h incubation, supernatants were collected and analysed for IL-8 concentration by enzyme-linked immunosorbent assay (ELISA). Data are expressed as pg/10⁵ cells. Statistical significance *versus* cells stimulated with TNF- α alone is indicated (analysis of variance and Dunnet's multiple comparison test, $n = 8$ –12).

intensity of the anti-ICAM-1 antibody was similar to that of the control antibody. After 20 h incubation with TNF- α , ICAM-1 expression was up-regulated on both bronchial (BEAS-2B and NHBE) and alveolar (A549) cells (Fig. 2a–c). Expression of VCAM-1 was markedly increased on BEAS-2B cells (Fig. 2d), while this cell adhesion receptor was not induced on A549 cells and only to a minor degree on NHBE cells (data not shown). Treatment with α -tocopherol 45 min before the stimulation with TNF- α resulted in concentration-dependent inhibition of ICAM-1 expression on both A549 and BEAS-2B cells as well as VCAM-1 on BEAS-2B cells. The most pronounced inhibition of ICAM-1 was observed at 50 μ M. In BEAS-2B cells the down-modulation of ICAM-1 was partially reversed at 100 μ M. Treatment with 50 μ M α -tocopherol resulted in decreased cell surface density of ICAM-1 to 59% on A549 cells, to 60% on BEAS-2B cells and to 59% on NHBE cells of cells activated with TNF- α alone. α -Tocopherol completely diminished the effect of TNF- α on NHBE cells and almost reduced the ICAM-1 expression to baseline level in BEAS-2B cells.

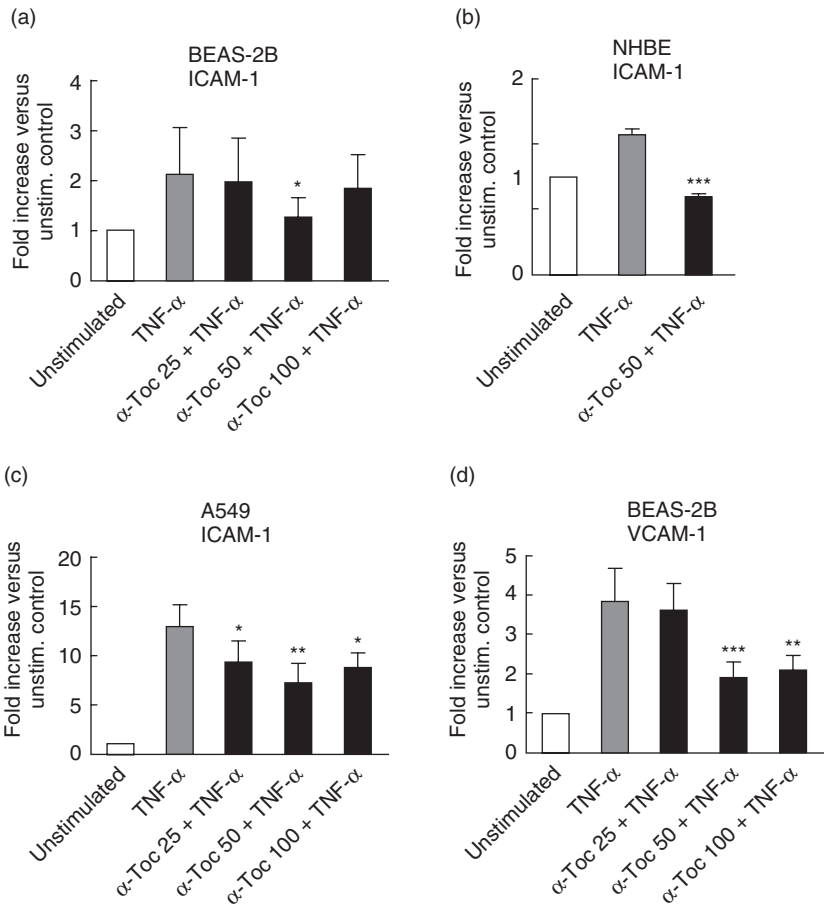


Fig. 2. Effect of α -tocopherol on cell surface expression of adhesion molecules intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cell line (BEAS)-2B (a), normal human bronchial epithelial cells (NHBE) (b), A549 (c) and vascular cell adhesion molecule (VCAM)-1 on BEAS-2B (d) cells. Cells were treated for 45 min with α -tocopherol in a concentration range of 25–100 μ M and thereafter stimulated for 20 h with 10 ng/ml tumour necrosis factor (TNF)- α . The surface expression of ICAM-1 and VCAM-1 was measured by flow cytometry. Data are presented as fold increase of median fluorescence of ICAM-1 or VCAM-1 expression *versus* unstimulated cells. Statistical significance *versus* cells stimulated with TNF- α alone is indicated (analysis of variance and Dunnet's multiple comparison test). $n = 4$ except for cells stimulated with TNF- α alone and α -tocopherol 50 in (a) and (c) ($n = 8$).

α -Tocopherol diminishes the adhesion of monocytes to lung epithelial cells

To determine the biological role of the down-modulation of cell adhesion molecules, we performed a cell adhesion assay using a Cr⁵¹ labelled monocyte cell line U937 and a monolayer of TNF- α -induced BEAS-2B cells. Stimulation with TNF- α 20 h prior to the assay clearly enhanced adhesion of the monocytes to the bronchial epithelial cells (Fig. 3). Incubation with α -tocopherol for 45 min before TNF- α stimulation reduced the adhesion of U937, especially at the 50 μ M concentration. Similar results were obtained with A549 cells (data not shown). Pretreatment of BEAS-2B cells with anti-ICAM-1 and anti-VCAM-1 antibodies completely abolished the TNF- α -induced adhesion of U937 cells, confirming that the cell adherence in our *in vitro* system is dependent on these surface receptors.

α -Tocopherol inhibits TNF- α -induced NF- κ B but not AP-1 activation

Because α -tocopherol down-regulated both cell surface expression of adhesion molecules and IL-8 production in lung epithelial cells, we investigated whether these effects could be explained by inhibition of key inflammatory

transcription factors. TNF- α induced strong activation of NF- κ B and AP-1 in both A549 and BEAS-2B cells 30 min after stimulation (Fig. 4). Treatment with α -tocopherol reduced the DNA binding of nuclear NF- κ B within the nucleus in A549 cells, with the most pronounced effect observed with 50 μ M (Fig. 4a). At this concentration, a similar effect was seen in BEAS-2B cells. Both 50 μ M and 100 μ M α -tocopherol significantly reduced DNA binding of NF- κ B in A549 cells. The 50 μ M concentration of α -tocopherol also reduced NF- κ B binding in BEAS-2B cells (Fig. 4c). The down-modulation of nuclear NF- κ B was followed by decreased levels of IL-8 and ICAM-1 mRNA 1.5 h later, supporting that the observed suppression of the transcription factor resulted in reduced gene transcription, although altered mRNA levels may also be reflected by changes in mRNA stability (data not shown). In contrast, 50 μ M α -tocopherol had no effect on AP-1 binding activity in either A549 or BEAS-2B cells compared with cells stimulated with only TNF- α (Fig. 4b).

α -Tocopherol inhibits TNF- α -induced ERK1/2 and p38 activation

To investigate the effect of α -tocopherol on MAPK signalling pathways activated by TNF- α , cell lysates were analysed by

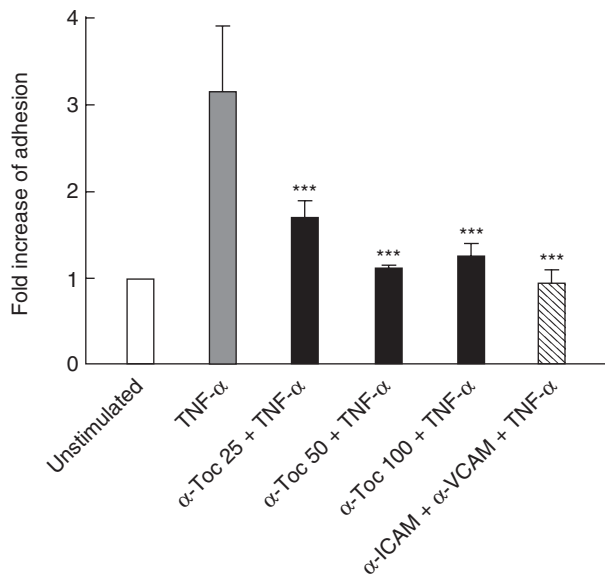


Fig. 3. Effect of α -tocopherol on monocyte adherence to tumour necrosis factor (TNF)- α -activated bronchial epithelial cell line (BEAS)-2B cells. ^{51}Cr -labelled U937 cells were added to BEAS-2B monolayer treated with α -tocopherol in a concentration range of 25–100 μM followed by stimulation with 10 ng/ml TNF- α for 20 h. After 45 min non-adherent cells were removed by washing and remaining cells were lysed with 5% Triton. The lysates were assayed for ^{51}Cr activity. Data are presented as fold increase of adhesion *versus* unstimulated cells. For comparison, the effect of pretreatment with antibodies against intercellular adhesion molecule-1 (α -ICAM) and vascular cell adhesion molecule-1 (α -VCAM) is also shown. Statistical significance *versus* cells stimulated with TNF- α alone is indicated (analysis of variance and Dunnet's multiple comparison test, $n = 6$).

immunoblot analysis using antibodies recognizing the phosphorylated active state of ERK1/2 and p38. The phosphorylated form of these MAPK were expressed only slightly in resting A549 and BEAS-2B cells, while stimulation with TNF- α for 15 min induced a clearly detectable increase of both phosphorylated-ERK1/2 and phospho-p38 in both cell lines (Fig. 5). Treatment with α -tocopherol reduced the phosphorylation of ERK1/2 55% in A549 and to 50% in BEAS-2B

cells of cells treated with TNF- α alone. Similarly, the phosphorylation of p38 was reduced in A549 by 25% and by 33% in BEAS-2B cells. α -Tocopherol almost completely attenuated the activation of ERK1/2 and p38 in BEAS-2B cells.

Effects of I κ B inhibitor on TNF- α -induced IL-8 and ICAM-1 expression

We used the specific I κ B inhibitor BAY 11-7082 to investigate if secretion of IL-8 and expression of ICAM-1 was dependent on the NF- κ B activation pathway in lung epithelial cells. Addition of this inhibitor diminishes the activation of NF- κ B by preventing phosphorylation of the inhibitory protein I κ B $_{\alpha}$ bound to NF- κ B. Cell cultures of A549 cells and BEAS-2B were incubated with BAY 11-7082 (8 μM and 1 μM , respectively) 60 min before exposed to 10 ng/ml TNF- α for 20–24 h. The production of IL-8 was measured in cell supernatants by ELISA and the cell surface expression of adhesion molecules were analysed by flow cytometry. The I κ B inhibitor almost completely inhibited TNF- α -induced secretion of IL-8 and expression of ICAM-1 in both cell lines, indicating that the induction of these inflammatory mediators is dependent on the NF- κ B activation pathway (Table 1).

Effects of MAPK and PKC inhibitors on TNF- α -induced IL-8 and ICAM-1 expression

To examine the intracellular signalling pathways involved in TNF- α -induced ICAM-1 expression and IL-8 secretion, the p38 inhibitor SB203580, the MEK inhibitor PD98059 and the PKC inhibitor Calphostin C were used. SB203580 acts by competitively inhibiting ATP binding to p38 [27]. PD98059 is a selective inhibitor of the MAPK kinases MEK1/2 which phosphorylates and activates ERK1/2 [23]. Calphostin C interacts with the PKC regulatory domain by competing at the binding site of diacylglycerol [28]. Cell cultures of A549 and BEAS-2B cells were incubated with either 1 μM SB2035190, 40 μM PD98059 or 0.5 μM Calphostin C for 45 min before stimulation with 10 ng/ml TNF- α for 24 h.

Table 1. Effect the of the inhibitor of kappa B (I κ B) (BAY 11-7082) on tumour necrosis factor (TNF)- α - induced interleukin (IL)-8 secretion and intercellular adhesion molecule-1 (ICAM-1) expression.

Cells	Treatment	IL-8	ICAM-1
		Fold increase <i>versus</i> unstimulated control	Fold increase <i>versus</i> unstimulated control
A549	Unstimulated	1.0	1.0
	TNF- α	4.6 \pm 0.4	5.5 \pm 0.1
	TNF- α + BAY 11-7082	1.1 \pm 0.1***	1.4 \pm 0.1***
BEAS-2B	Unstimulated	1.0	1.0
	TNF- α	35.1 \pm 3.5	3.2 \pm 0.1
	TNF- α + BAY 11-7082	6.0 \pm 0.9***	1.3 \pm 0.1***

*** $P < 0.001$ (P -values are indicated when the I κ B inhibitor BAY 11-7082 + TNF- α treatment was significantly different from treatment with TNF- α alone). Mean values \pm s.d. are indicated, $n = 4$. BEAS: bronchial epithelial cell line.

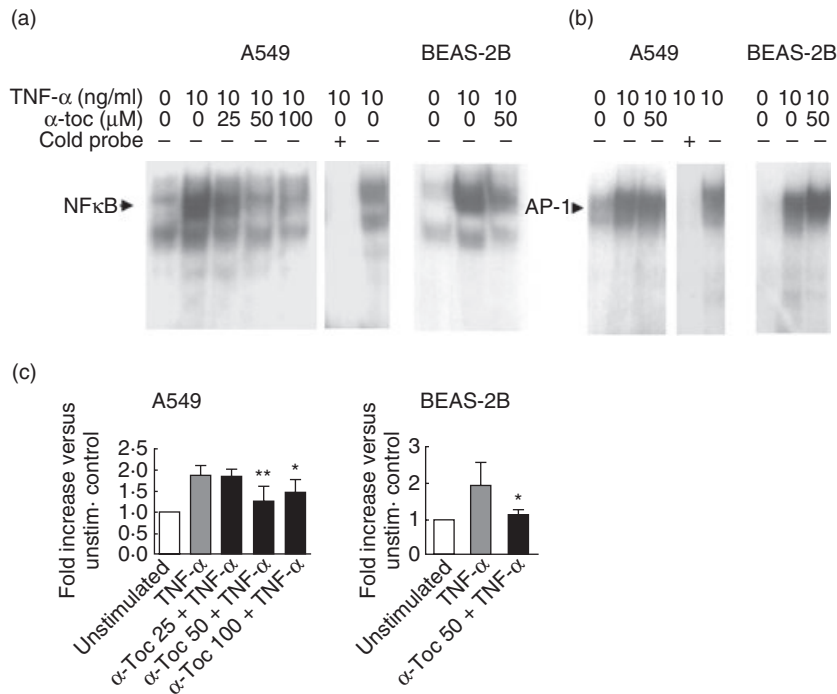


Fig. 4. Effect of α -tocopherol on nuclear factor kappa B (NF- κ B) and activator protein-1 (AP-1) activity in A549 and bronchial epithelial cell line (BEAS)-2B cells. Cells were treated for 45 min with or without α -tocopherol and stimulated thereafter for 30 min with 10 ng/ml tumour necrosis factor (TNF)- α . Nuclear extracts were analysed using electrophoretic mobility shift assay (EMSA). Five μ g nuclear extract/lane was loaded. Representative autoradiograms of NF- κ B (a) and AP-1 (b) activity are shown. The results from unstimulated cells are shown in the left lane of each gel. Control experiment using excess of unlabelled NF- κ B or AP-1 verifying that the binding of [32 P]-labelled oligonucleotides was specific are shown to the right in EMSA analysis of A549 cells. Intensity of bands, corresponding to NF- κ B, was quantified using an image analysis system (c). Data are expressed as fold increase in intensity versus unstimulated cells. Statistical significance versus cells stimulated with TNF- α alone is indicated (analysis of variance and Dunnet's multiple comparison test $n = 5$).

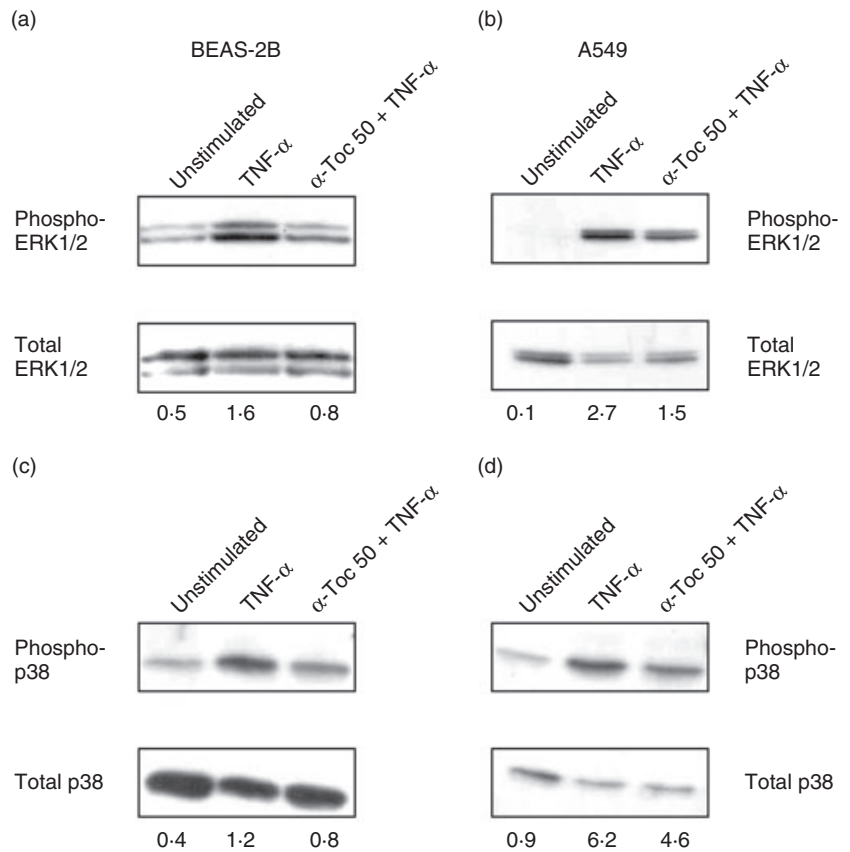
The production of IL-8 was measured in cell supernatants by ELISA (Fig. 6) and the cell surface expression of adhesion molecules were analysed by flow cytometry (Fig. 7). All three inhibitors decreased IL-8 secreted from BEAS-2B cell cultures to 40–60% of cells stimulated by TNF- α only (Fig. 6). In A549 cells, the ERK1/2 inhibitor PD98059 almost completely abolished the TNF- α -induced IL-8 production while the p38 inhibitor reduced the cytokine concentration to 50%, and the PKC inhibitor Calphostin C reduced the concentration to 70%. The ICAM-1 cell surface expression was not affected by the MAPK inhibitors neither in A549 or BEAS-2B cells, compared with cells incubated with TNF- α only (Fig. 7). Calphostin C showed no significant effect on ICAM-1 expression on BEAS-2B while this inhibitor down-modulated the expression of ICAM-1 to 60% on the A549 cells. For comparison, samples treated with 50 μ M α -tocopherol were included in the experiment and the effects are shown in Figs 6 and 7.

Discussion

In the present study, we investigated the effects of α -tocopherol on the responsiveness of human lung epithelial

cells to a proinflammatory signal, TNF- α . We demonstrated that exogenously administered α -tocopherol inhibited production of the neutrophil chemoattractant IL-8, as well as down-regulating the expression of cell adhesion molecules in both A549 and BEAS-2B cell lines and untransformed primary cells. The inhibition was concentration-dependent, with the most pronounced effect at 50 μ M. Furthermore, we demonstrated that the down-modulation of cell adhesion molecules were reflected by a reduced ability of BEAS-2B and A549 cells to bind leucocytes *in vitro*. Further increase of the concentration to 100 μ M did not, however, result in enhanced inhibition. Instead, a tendency towards reversed effect was observed. Such a response might be explained by disturbance of the cellular balance of water-soluble and lipophilic antioxidants at very high α -tocopherol concentrations, leading to action as an intracellular oxidant. Our observation is in agreement with a previous study by Shang *et al.*, reporting that > 80 μ M vitamin E enhances the toxicity of hydrogen peroxide on epithelial cells [29]. However, it should be noted that from our data it is evident that responsiveness to high concentrations of α -tocopherol was not equal in A549 and BEAS-2B cells, and also that we observed a tendency towards differential effects on IL-8 and ICAM-1 expression.

Fig. 5. Effect of α -tocopherol on tumour necrosis factor (TNF)- α -induced extracellular signal-regulated kinase (ERK)1/2 (a and b) and p38 (c and d) activation in bronchial epithelial cell line (BEAS)-2B (a and c), A549 cells (b and d). Cells were treated for 45 min with 50 μ M α -tocopherol or solvent before stimulation with 10 ng/ml TNF- α for 15 min. Cells were lysed and 25 μ l of cell lysates were loaded onto 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer to Immobilon-P membranes. Immunoblotting was performed with anti-phospho-specific mitogen-activated protein kinases (MAPK) antibody or an antibody recognizing total MAPK, followed by incubation with horseradish peroxidase-conjugated secondary antibody. The bands were visualized with the enhanced chemiluminescence (ECL) Western blot detection system. The double bands in (a) and (b) indicate the ERK1 (p42) and ERK2 (p44) isoforms, respectively. Numbers at the bottom of the lanes are densitometry ratio of phosphorylated MAPK *versus* total MAPK corrected for variations in protein loading. The blots are representative of three independent experiments.



In our experimental system cells were stimulated with TNF- α , a proinflammatory cytokine derived from many inflammatory cells, such as monocytes and macrophages. This activation of lung epithelial cells is compatible with two major scenarios of acute airway inflammation *in vivo*: (1) macrophage ingestion of inhaled compounds leading to a vigorous cytokine secretion from these cells to airspaces and (2) fulminant inflammatory response in lung interstitium following a systemic trauma, i.e. the setting of indirect ARDS. Such responses can be provoked in laboratory animals by exposure to compounds that activate innate immune mechanisms. We have previously utilized C57BL/6 mice to study early inflammatory events in airways following inhalation of aerosolized *E. coli* LPS [30]. In these mice, engagement of Toll-like receptor 4 by LPS [31] induces onset of proinflammatory cytokines and chemokines in lung tissue within 2 h leading to accumulation of neutrophils in airspaces [30]. These sequestered neutrophils exhibit an enhanced ability to produce reactive oxygen metabolites [32], and concurrent with the airway inflammation, lung oedema and signs of epithelial cell injury were observed [12]. Using this model, we have demonstrated that exogenously administered α -tocopherol reduces neutrophilia and prevents lung injury [12]. Chow and coworkers have suggested three possible mechanisms for the action of α -tocopherol *in vivo*: (i) prevention of neutrophil adhesion by abrogating the

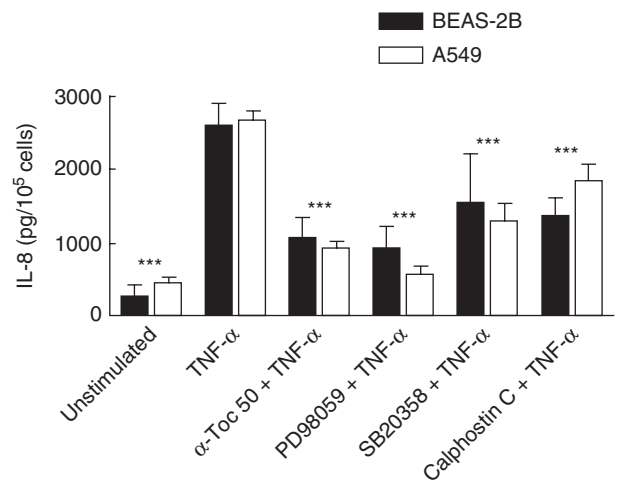


Fig. 6. Effects of extracellular signal-regulated kinase (ERK)1/2 (PD98059), p38 SD203580 or protein kinase C (PKC) (Calphostin C) inhibitors on tumour necrosis factor (TNF)- α -induced interleukin (IL)-8 production. Bronchial epithelial cell line (BEAS)-2B or A549 cells were incubated with 40 μ M PD98059, 1 μ M SB203580 or 0.5 μ M Calphostin C. For comparison, treatment 50 μ M α -tocopherol was included in the experiment. After 24 h, supernatants were collected and the concentration of IL-8 was measured by enzyme-linked immunosorbent assay (ELISA). Statistical significance of both cell lines *versus* cells stimulated with TNF- α alone is indicated (analysis of variance and Dunnet's multiple comparison test, $n = 12$).

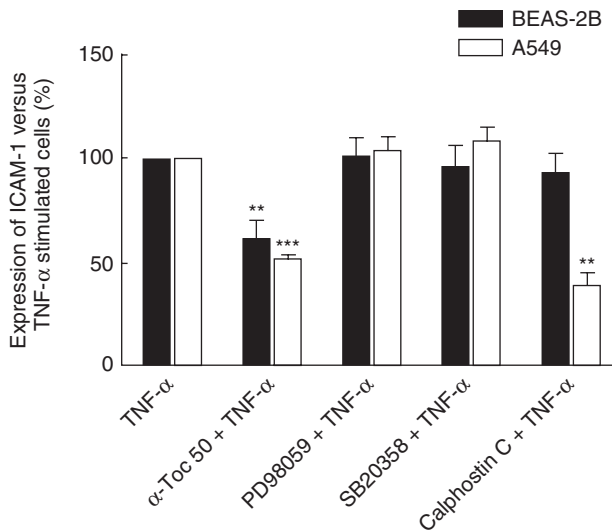


Fig. 7. Effects of extracellular signal-regulated kinase (ERK)1/2 (PD98059), p38 (SP203580) and protein kinase C (PKC) (Calphostin C) inhibitors on tumour necrosis factor (TNF)- α -induced cell surface expression of intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cell line (BEAS)-2B and A549 cells. Cells were preincubated with 40 μ M PD98059, 1 μ M SB203580 or 0.5 μ M Calphostin C. For comparison, treatment with 50 μ M α -tocopherol was included in the experiment. The surface expression of ICAM-1 was measured by flow cytometry. Results are expressed as percentage of remaining cell surface expression of ICAM-1 *versus* TNF- α -stimulated cells. Expression on cells stimulated with TNF- α alone was given the value 100%. Statistical comparisons were performed using one-sample *t*-test *versus* 100% ($n = 4$).

expression of adhesion molecules on the surface of endothelial cells and/or neutrophils; (ii) impaired neutrophil binding to the endothelium due to changes in the topographic distribution or binding affinities of the surface adhesion molecules; and (iii) impaired neutrophil transmigration due to changes in the membrane stability of the migrating neutrophils, the endothelial and/or the epithelial cells [33].

In addition to the direct effects on cell membrane morphology, it is evident from our data that α -tocopherol interacts with intracellular activation pathways controlling inflammatory responses. Our results, together with previously reported data, indicate clearly that IL-8, as well as the cell adhesion receptor ICAM-1, are transcriptionally regulated by NF- κ B [34,35]. Thus, it can be assumed that the observed down-modulation of this transcription factor accounts at least partially for the decreased responses observed in the α -tocopherol-treated cells. Consistent with this, α -tocopherol has been shown to reduce NF- κ B transcriptional activity in the THP-1 monocyte cell line [26]. In bronchial epithelial cells, engagement of TNF- α with the 55-kDa TNF- α receptor (TNF-RI) activates protein kinase C (PKC) through a pathway involving phospholipase C-mediated hydrolysis of phosphatidylcholine to diacylglycerol [1]. Krunkosky *et al.* suggested that activation of PKC is

an upstream signalling event controlling nuclear translocation of NF- κ B, although this linkage was not demonstrated conclusively [1]. If this putative pathway is present in the epithelial cells, our data on DNA binding of NF- κ B could be assigned as a downstream effect of the previously described dephosphorylation of PKC [36]. In addition, Sabat *et al.* have demonstrated that depletion of vitamin E increased ICAM-1 expression on type II cells. This effect was assumed to be mediated by PKC activation, as the redox status was not affected [37]. However, other investigators have suggested that down-regulation of ICAM-1 and integrins by α -tocopherol can occur in a PKC-independent manner [23,24]. In our study, we used the specific PKC inhibitor Calphostin C to investigate the PKC dependency of the TNF- α -induced inflammatory response. This inhibitor clearly diminished ICAM-1 expression on the type II alveolar A549 cells, while the expression on bronchial BEAS-2B cells was unaffected. The differential inhibition of ICAM-1 could be due hypothetically to expression of different PKC isoforms in A549 and BEAS-2B cells. Notably, this observation contrasts with the reduced IL-8 production in both A549 and BEAS-2B cells after treatment with Calphostin C. Treatment with α -tocopherol did not result in such differential effects, indicating that the actions of this vitamin do not mimic those of PKC inhibitors, at least with regard to intervention of ICAM-1 expression on BEAS-2B cells.

In this study, we also demonstrated that α -tocopherol down-modulates phosphorylation of ERK1/2 and p38. These protein kinases belong to a family of MAPK used by human cells to transduce extracellular signals into a cellular response. ERK1/2 differs from the two other major members, p38 and JNK, by the lack of strong linkage with induction of proinflammatory responses [37] but, rather, is thought to play a role regulating proliferation, transformation and differentiation [38,39]. Both p38 and JNK phosphorylation has been related clearly to NF- κ B and AP-1 activation partly by converging pathways, while the linkage between ERK1/2 and these transcription factors has been elusive [40–46]. Other studies have shown that inhibition of the ERK pathway diminishes TNF- α production in monocytes and macrophages [47,48] and IL-8 secretion in respiratory epithelial cells [49,50]. We demonstrated that the ERK inhibitor PD98059 blocks IL-8 production both in the A549 and BEAS-2B cell lines, further supporting a role of ERK1/2 activation in the expression of proinflammatory cytokines by lung epithelial cells. The effect on ERK1/2 phosphorylation by α -tocopherol can be due hypothetically to down-regulation of upstream signalling events. Two such major pathways leading to ERK phosphorylation have been described: (1) the PKC signalling pathway and (2) activation of RAS through receptor tyrosine kinase with subsequent phosphorylation of RAF and MEK1/2 [51]. Although it appears that these ERK activation pathways are independent, it should be emphasized that signal transduction mechanisms are generally cross-regulated in an intricate network

[51,52]. Thus, it is not possible to delineate exactly the intracellular mechanisms leading to activation of this MAPK. Our observation that both Calphostin C and PD98059 inhibit IL-8 expression in A459 cells indicates that the IL-8 production is dependent on both ERK and PKC activation. In contrast to IL-8, the induction of ICAM-1 was independent of both ERK and p38 activation. Comparing the effects of α -tocopherol with that of the different inhibitors revealed that α -tocopherol, differently to the specific MAPK and PKC inhibitors, consistently down-modulated ICAM-1 expression in the two cell lines. This implicates a broader action of α -tocopherol, probably targeting more than one signal transduction mechanism.

In conclusion, the action of α -tocopherol on activated epithelium can protect cells from oxidative stress and excessive inflammation by inhibition of signal transduction pathways. We have demonstrated that α -tocopherol reduces ERK1/2 and p38 phosphorylation, as well as DNA binding of NF- κ B in bronchial and alveolar epithelial cells. This was followed by diminished secretion of IL-8 and down-regulation of leucocyte adhesion by the epithelial cells. These results add new insights to the mechanisms of protection against inflammatory responses in the lung epithelium by α -tocopherol.

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