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Stimulation of stress-activated but not mitogen-activated protein kinases by tumour necrosis factor receptor subtypes in airway smooth muscle

Shona M. McFarlane[a], Orla J. Jupp[a], Hannah J. Cobban[a], Irene Hunter[a], Helen M. Anderson[a], Peter Vandenabeele[b], Graeme F. Nixon[a], David J. MacEwan[a,*]

[a]*Department of Biomedical Sciences, Institute of Medical Sciences, Foresterhill, University of Aberdeen, Aberdeen, AB25 2ZD, UK*
[b]*Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology, University of Gent, Ledeganckstraat 35, B-9000 Ghent, Belgium*

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Abstract

The multifunctional cytokine tumour necrosis factor-α (TNF) displays many physiological effects in a variety of tissues, especially proliferative and cytotoxic actions in immunological cells. Recently, we uncovered an important new mechanism by which TNF can sensitise airway smooth muscle (ASM) to a fixed intracellular Ca^{2+} concentration which *in vivo* would produce a marked hypercontractility of the airways. Here, we report that both 50–60 kDa type I TNFR (TNFR1) and 70–80 kDa type II TNFR (TNFR2) receptor subtypes were expressed in ASM cells and selectively activated the stress kinases, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (p38 MAPK). However, TNF caused no activation of p42/p44 MAPK or cytosolic phospholipase A_2 activity. In contrast, TNF stimulation of the TNFR1, but not the TNFR2, elicited nuclear factor-κB transcription factor function, a species known to be important in mediation of certain inflammatory cellular responses. This is the first report of TNF receptor subtypes in ASM cells causing selective kinase activation, which may prove important in therapeutic strategies for treating immune airway disorders such as chronic obstructive pulmonary disease and asthma.

Keywords: Tumour necrosis factor; Airways; Smooth muscle; Hypercontractility; Signalling; Asthma

1. Introduction

Inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease are characterised by a prolonged inflammatory reaction resulting from exposure to spasmogen [1,2]. Initially, airflow is retarded by blockage of the airways with an excessive build-up of mucus, although difficulty in breathing is mostly manifested by a reduced lung capacity caused by bronchial smooth muscle hyperconstriction, resulting in eventual morphological alterations in airway anatomy [1,3].

The precise biochemical nature of the onset of airway hyperresponsiveness in diseases such as asthma is not fully understood. It is known to involve inflammatory mediators including endothelins, bradykinin, a variety of cytokines, and lipid products such as leukotrienes and thromboxanes [1,2]. Identification of the most crucial of these components remains a priority in attempts to manage acute and chronic inflammatory airway disorders. Current therapies are restricted to broad spectrum anti-inflammatory steroidal or antihistamine treatments, airway smooth muscle relaxants such as β2-adrenoceptor agonists, or inhibitors of leukotriene production [4]. These therapies are used to control the symptoms of asthma rather than directly treating the cause of the disease.

Recent work has identified TNF as a cytokine that is important in the hyperreactivity of airway tissue [5–7]. Indeed, a possible site for pharmacological intervention is to block the effects of TNF in airways [8–10], proving the efficacy and clinical importance of TNF's contribution towards asthmatic symptoms. The mechanisms by which TNF can induce ASM hypercontractility may involve

* Corresponding author. Tel.: +44-1224-273.154; fax: +44-1224-273.019.

E-mail address: david.macewan@abdn.ac.uk (D.J. MacEwan).

Abbreviations: ASM, airway smooth muscle; $cPLA_2$, cytosolic phospholipase A_2; DMEM, Dulbecco's modified Eagle's medium; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor-κB; PDBu, phorbol-12,13-dibutyrate; R1-TNF, R32W,S86T-TNF; R2-TNF, D143N,A145R-TNF; TNF, tumour necrosis factor-α; TNFR, TNF receptor; TNFR1, 50–60 kDa type I TNFR; TNFR2, 70–80 kDa type II TNFR; and TRAF, TNF receptor-associating factor.

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increased intracellular calcium at some stage [11,12], although this evidence would apply to longer exposure times (18 hr). We have uncovered a novel mechanism by which TNF can cause hypercontractility in ASM with much shorter exposure times (min) to TNF [13]. Activation of tracheal or bronchial smooth muscle leads to a sustained ability of TNF to sensitise the smooth muscle myofilaments to Ca^{2+} and was evident with short incubation times. This so-called 'Ca^{2+} sensitisation' phenomenon has already been documented in smooth muscle to a range of classical contractile agonists, but this is the first report of a cytokine receptor mediating this effect. Moreover, TNF is a pure sensitising agent, as it does not contract by itself, but rather enhances contractile responses to other Ca^{2+}-releasing stimuli [13].

TNF actions are mediated by two receptor subtypes, TNFR1 and TNFR2 (also known as p55TNFR and p75TNFR) [14]. The biochemical machinery employed by these TNFRs is still being uncovered. It appears that activated TNFR subtypes stimulate a wide array of signalling macromolecules, but they do not appear to induce the release or subcellular redistribution of Ca^{2+} ions. TNFRs achieve their biochemical actions through interacting with a range of TRAFs that bind selectively to the TNFRs and signal for several enzymatic activities [15,16]. In this way, TNFRs can activate lipases such as sphingomyelinase and phospholipases C, kinase pathways such as the mitogen-, ceramide- and stress-activated pathways, as well as Rafl, protein kinase C cascades, while transcription factor activities such as NFκB are also activated [14]. These biochemical pathways ultimately control the cellular responses in cells [17] depending on the biochemical status of the cell and the TNFR subtypes expressed [14]. Almost nothing is known about the signalling processes by which TNF has its effects in ASM. This study investigates TNFR expression and extracellular signal-regulated kinase (ERK) signalling in ASM cells.

2. Materials and methods

2.1. Materials

Recombinant human TNF was purchased from R&D Systems. The cytokine's biological activity was confirmed by measurement in the L929 cytotoxicity assay when comparing cytotoxic activity of TNF standards (kindly provided by Dr. Meenu Wadhwa, National Institute of Biological Standards and Controls, Potters Bar, UK). Each batch of TNF was confirmed to have at least 2×10^7 WHO U/mg. Phospho-specific MAPK antisera were purchased from New England Biolabs. Pan-MAPK antiserum used as a control was obtained from Santa Cruz Biotechnology. $cPLA_2$ antiserum was acquired from The Binding Site Ltd. ^{125}I-TNF (specific activity 500–100 Ci/mmol), [γ-^{32}P]ATP (specific activity $>$ 3000 Ci/mmol), and glutathione-Sepharose 4B beads were purchased from Amersham Pharmacia Biotech. c-Jun (5–89)–glutathione *S*-transferase chimeric-expressing bacteria were generously provided by Drs. James Woodget (University of Toronto, Canada) and Robin Plevin (University of Strathclyde, UK). All other materials were from BDH/Merck Ltd. or from the Sigma Chemical Co. and were of the highest grade obtainable.

2.2. Cell culture

Male Duncan–Hartley guinea pigs (300–350 g) were killed by cervical dislocation and the trachea removed and cleared of fat, connective tissue, and the epithelium. The central longitudinal section of the smooth muscle was removed and digested over 4–5 hr in digest mix, consisting of 1 μg/mL of type II collagenase, 0.2 mg/mL of type IV elastase, and 50 μL/mL of soybean trypsin inhibitor in serum-free DMEM. The smooth muscle was agitated every 30 min during digestion. Once digested, the tissue was transferred to a flask and cultured with DMEM + 10% foetal bovine serum, 2 mM L-glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. After 24-hr incubation, medium was replaced with 20% foetal bovine serum-containing medium. Once cells had attached and started to proliferate, medium was changed back to 10% foetal bovine serum DMEM. Once confluent, cells were split in a 1:3 dilution. Experiments were not carried out on cells past the sixth passage.

2.3. ^{125}I-TNF binding analysis

Mutational analysis of human TNF revealed that certain mutations of the wild-type sequence could enable the mutated protein to selectively bind to either of the TNFR subtypes. The specific double mutation of R32W,S86T (termed R1-TNF) allows selective activation by this mutant protein ('mutein') of the TNFR1 only, whereas the D143N,A145R (termed R2-TNF) double mutation allows selective activation of the TNFR2 subtype only [18,19]. Specific binding analysis was performed essentially as described [20,21], using ^{125}I-TNF and 200-fold excess non-radiolabelled TNF for total and non-specific binding determinations, respectively. TNFR1-specific binding was determined with a 200-fold excess of R2-TNF mutein; conversely, TNFR2-specific binding was determined in the presence of R1-TNF. Binding to confluent ASM cells in serum-containing medium (100 μL/well) was performed on ice in 96-well plates, and preincubated for 40 min with cold TNF, R1-TNF, or R2-TNF before addition of 2×10^5 cpm/well of ^{125}I-TNF. Plates were incubated on ice for a further 1 hr, then washed with 3×200 μL/well of PBS + 0.1% BSA and counted on a Wallac 96-well counter.

2.4. Fluorescence-activated cell sorting (FACS) analysis

ASM cells were grown to 70–90% confluency and dissociated with 2 mL of trypsin-free cell dissociation solution (Sigma). Cells were washed and then resuspended in serum-free DMEM to give a cell population of 5×10^6 cells. A 200-μL aliquot was incubated on ice for 1 hr in a 1:200 dilution of R&D Systems mouse anti-rodent TNFR1 or TNFR2 primary antibody (or pan-MAPK immunoglobulin G [IgG] antibody for control assessments). Cells were centrifuged at 800 *g* for 2 min and then resuspended again in 200 μL serum-free DMEM with a 1:50 dilution of fluorescein isothiocyanate (FITC)-labelled anti-sheep/goat antibody (Scottish Antibody Production Unit, Carluke, UK) for a 1-hr incubation on ice. Prior to FACS analysis, the cells were washed three times and resuspended in PBS + 2% foetal bovine serum. FACS analysis was performed in a Becton Dickinson FACScaliber.

2.5. Confocal fluorescence microscopy

ASM and HeLa cells were treated for 0, 30, and 60 min with either TNF-α (50 ng/mL) or ionomycin (1 μM). Both cell types were then fixed with ice-cold methanol for 20 sec and washed with PBS prior to labelling for 60 min with antibody ($cPLA_2$) at a 1:100 dilution in Krebs solution (NaCl 137.4 mM, KCl 5.9 mM, $CaCl_2 . (6H_2O)$ 1.2 mM, $MgCl_2 . (6H_2O)$ 1 mM, HEPES 11.6 mM, glucose 11.5 mM). Primary antibody was removed and replaced by a 1:100 dilution of FITC-labelled anti-sheep/goat antibody. Cells were incubated in the dark at room temperature for a further 1 hr and washed 3 times in Krebs solution prior to visualisation of the labelled antibody. Confocal laser microscopy was performed on a Bio-Rad μradiance system measuring fluorescence at green/blue wavelengths (480–520 nm). Labelling of ASM cells with the anti-rodent TNFR1 and TNFR2 antisera was performed as above, except that the preparation was not fixed but incubated at 4°.

2.6. Western analysis

Cell monolayers were lysed with 2% SDS, 70 mM Tris.HCl (pH 6.8), nucleic acids were sheared with a 22 gauge needle, and the cell monolayers then boiled for 15 min to denature proteins and nucleic acids. Whole cell protein concentrations were determined by Lowry protein assay (Bio-Rad), then supplemented with 6× loading buffer (60% glycerol, 12.5% β-mercaptoethanol, 1% bromophenyl blue) as described [22]. After 12% SDS/PAGE, proteins were transferred to nitrocellulose (Costar) and equal loading and transfer then determined with reversible protein stain solution (0.1% Ponceau S in 3% trichloroacetic acid). After blocking for 1 hr with PBS, 0.5% Tween 20, and 5% non-fat dried milk, primary antibody binding (1:1000 of antiserum in PBS + 0.5% Tween, 2 hr) was followed by 3 × 5-min washes in PBS/Tween and a 1-hr incubation in PBS/Tween with 1:10,000 of biotinylated donkey anti-rabbit antibody. Three further washes were followed by a 1-hr incubation with 1:10,000 of streptavidin-conjugated horseradish peroxidase. After further washes, specific antibody interactions were detected by enhanced chemiluminescence.

2.7. c-Jun N-terminal kinase (JNK) activity

JNK activity was measured by assessing phosphorylation of its substrate c-Jun (5–89) linked to a glutathione *S*-transferase (GST) fusion protein essentially as described [21]. Subconfluent cells were treated for a period of 15 min. After stimulation, cells were lysed in solubilisation buffer (20 mM HEPES [pH 7.7], 50 mM NaCl, 0.1 mM EDTA, and 1% Triton X-100) with protease and phosphatase inhibitors. The cellular extracts were then affinity-precipitated with a slurry of c-Jun–GST and glutathione-linked Sepharose beads. Kinase reactions (30°) were initiated by the addition of kinase buffer (25 mM HEPES [pH 7.6], 20 mM $MgCl_2$, 0.1 mM Na_2VO_4, 5 mM β-glycerophosphate and 2 mM dithiothreitol) 25 μM ATP, and 1–2 μCi of [γ-^{32}P]ATP. Reactions were stopped after 30 min by the addition of 6 × Laemmli SDS sample buffer, then boiled for 4 min. After 12% SDS–PAGE, the phosphorylated c-Jun fusion protein was visualised by autoradiography and quantified on a Bio-Rad densitometer.

2.8. NFκB-luciferase reporter activity measurements

Cultured ASM cells were aliquoted into 6-well culture dishes at a density of 1×10^5 cells/mL of culture medium. When the cells were around 50% confluent, 1 μg/well of 3 × NFκB–luciferase reporter construct DNA [23] was introduced into the cells by the lipofectamine (GIBCO BRL) transfection protocol for 3 hr before reintroduction into serum-containing media. After an overnight incubation, the culture medium was replaced with fresh DMEM containing 10% foetal calf serum. Forty-eight hours posttransfection, the cells were treated for 5 hr with stimulus before being washed twice in ice-cold PBS. A 5-min incubation on ice followed, using 200 μL/well of ice-cold lysis buffer (25 mM Tris phosphate [pH 7.8], 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton X-100, 15% glycerol). The cell extracts were then scraped into 1.5-mL Eppendorf tubes and centrifuged to pellet debris. One hundred microlitres of the supernatant was used to measure luciferase induction by injecting it with an equal volume of luciferase buffer (lysis buffer containing 1 mM ATP, 0.25 mM luciferin, 1% BSA) in a Berthold LB9501 Lumat luminometer.

2.9. Cytotoxicity measurements

Cultured ASM cells were aliquoted into 96-well culture plates at a density of 1×10^5 cells/mL of culture medium (100 μL/well) as described [24]. After 24-hr growth, the plates were treated with the required combination of agents

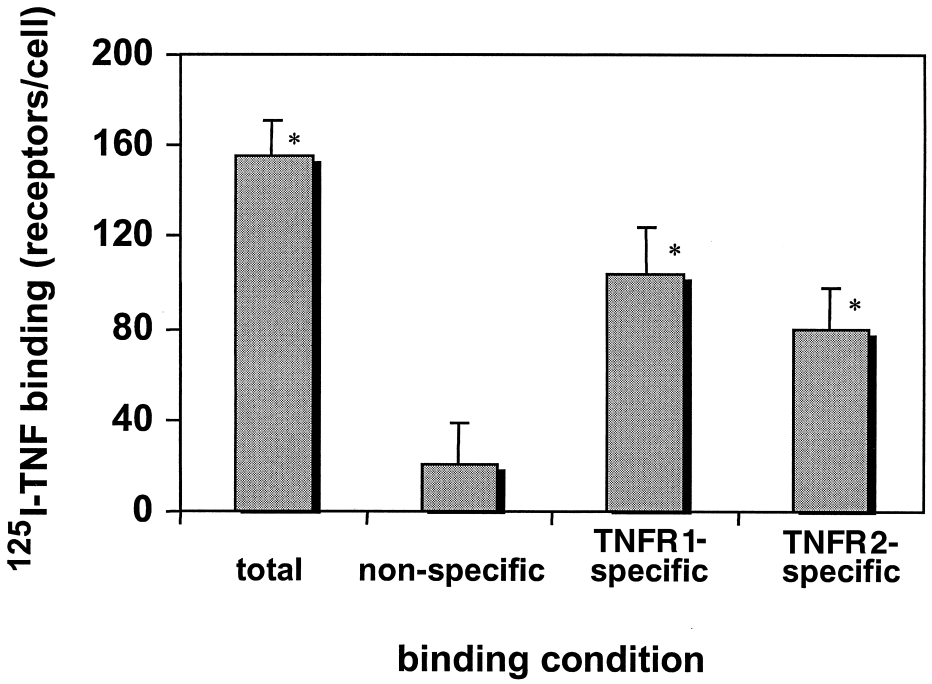

Fig. 1. ^{125}I-TNF binding to cultured airway smooth muscle cells. Cultured ASM cells were analysed for specific ^{125}I-TNF binding as described in the Methods section. The TNFR1 and TNFR2 subtype composition of the TNFR was determined in the presence of R2-TNF and R1-TNF, respectively. Non-specific binding was determined in the presence of excess unlabelled wild-type TNF. The data represent the means ± SD of quadruplicate determinations with a representative experiment shown. Statistical significance from control values ($P < 0.05$, Student's *t*-test) are shown (*).

and incubated for a further 24 hr. Colourimetric determination of the attached cell number was performed by washing the wells with PBS and fixing the remaining attached cells with PBS + 10% formaldehyde for 20 min. After another PBS wash, fixed cells were stained with 0.1% crystal violet, 18% ethanol in PBS for 30 min. Plates were flushed 4 times in water and dried overnight. Each well was eluted with 100 μL of 50% 0.1 M sodium citrate (pH 4.2), 50% ethanol for 30 min before colourimetric intensity was read at 570 nm.

3. Results

Fig. 1 reveals that cultured guinea pig ASM cells did indeed possess specific TNFRs. These cells expressed approximately 150 receptors/cell as revealed by radioligand binding analysis. This is a relatively low level of TNFR expression when compared to other cell types that endogenously express the receptor: HeLa human cervical epithelial cells, for example, express some 3000 receptors/cell [25]. The proportion of the receptors which are of the two TNFR subtypes is revealed by competition with subtype-specific TNF mutant proteins ('muteins') [20]. It was found that there were approximately equal proportions of the TNFR1 and TNFR2 subtypes in ASM (Fig. 1). This was confirmed in anti-rodent antibody binding studies using flow cytometry fluorescence-activated cell sorting (FACS) (Fig. 2). Here, it was seen that the two TNFR subtypes were present in approximately equal proportions on populations on cultured ASM cells, as indicated by the rightward shifts in specific antisera binding. The subcellular distribution and relative proportion of the TNFR1 and TNFR2 were revealed in confocal fluorescence microscopy experiments, indicating plasma membrane (non-nuclear) distribution of the TNFRs (Fig. 2). Comparisons within the same experiments using other cells that express TNFR subtypes abundantly (e.g. rodent L929 cells, data not shown) showed a similar receptor distribution, thereby supporting the evidence that ASM cells express both TNFR subtypes.

Activated TNFRs achieve their cellular effects through activation of a multitude of kinase pathways. We investigated here the capability of TNFRs expressed in ASM to activate extracellular signal-regulated kinase pathways. Activation by TNF of the stress-activated kinase JNK was also performed in cultured ASM cells. Studies with phospho-specific JNK antisera were futile due to the extremely limited nature of these antibodies for Western analysis. We therefore performed a specific radioactivity assay that revealed a rapid and transient activation by TNF of JNK (Fig. 3). The time-course of activation of JNK by TNF was maximal at 5–15 min, and suggests that this stress kinase pathway may somehow be involved in the rapid onset of the TNF-induced Ca^{2+} sensitisation and hypercontractility observed in TNF-stimulated ASM tissue [13]. Using phospho-specific antisera, we also determined that TNF treatment led to activation of the p38 MAPK pathway (Fig. 3). The activation of this stress kinase pathway occurred in a time-dependent manner with a lag period of around 30 min after

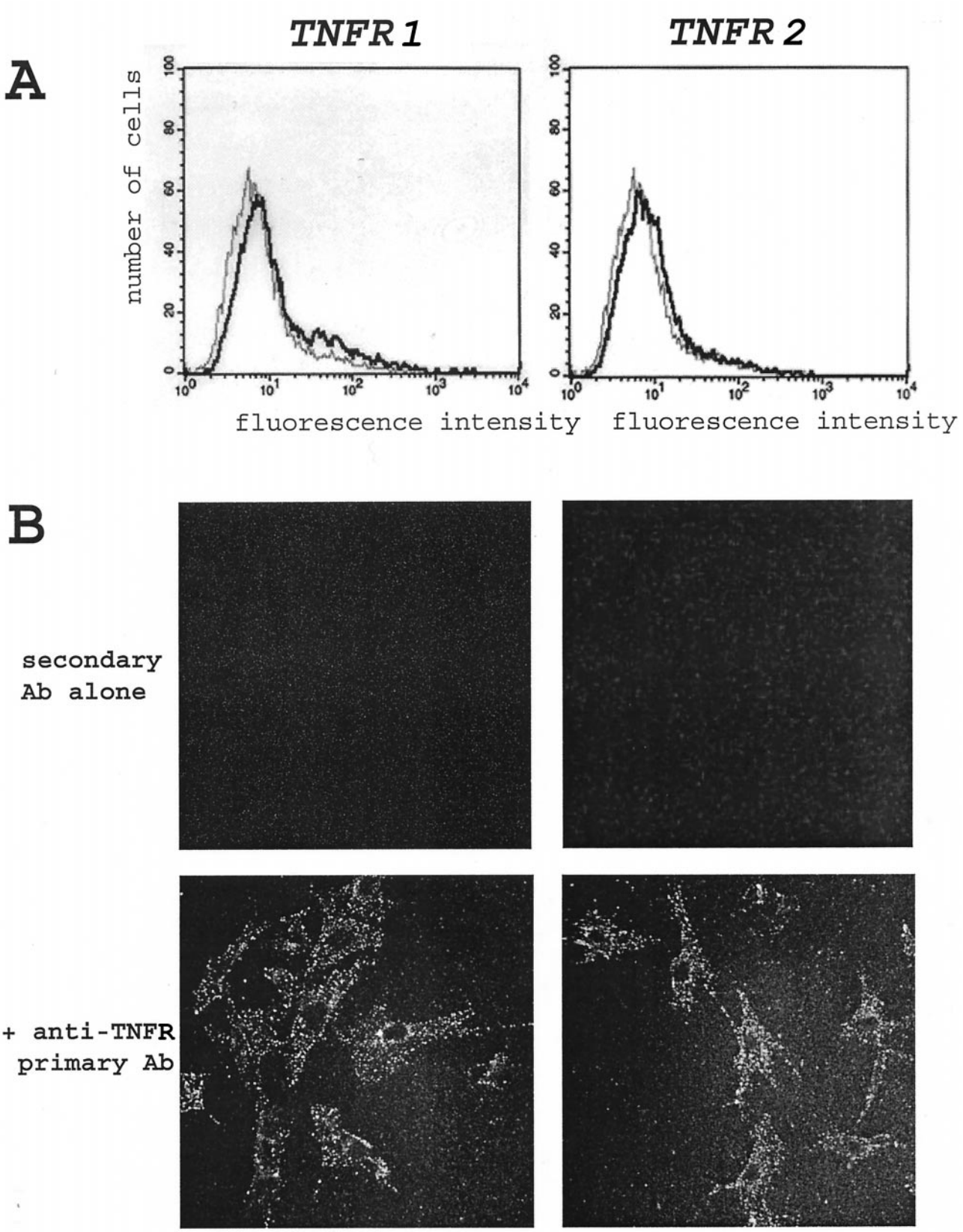

Fig. 2. TNFR subtype immunoreactivity on cultured ASM cells. (A) FACS analysis of the TNFR subtype on ASM cell populations. Comparisons of antisera binding of primary anti-TNFR specific (darker lines) or control antisera are shown (thin lines). Rightward shifts in the curves along the fluorescence intensity axis indicate antibody-specific binding. The results are from a representative experiment of at least two other independent determinations with similar findings. (B) Laser confocal immunofluorescence of TNFR subtypes on cultured ASM cells analysed for specific TNFR1 and TNFR2 subtype binding as described in the Methods section. Shown are typical images from two independent experiments.

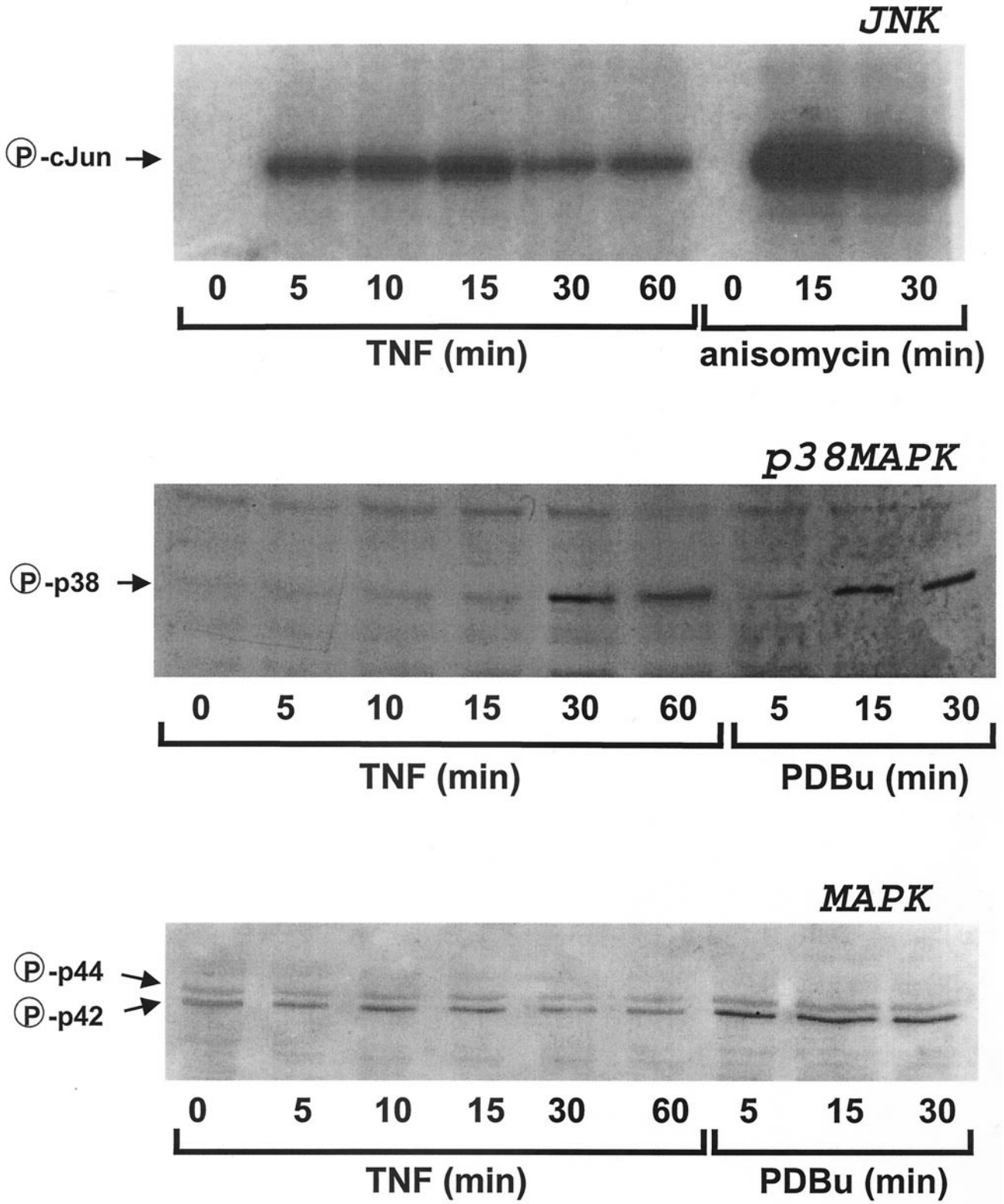

Fig. 3. TNF-induced JNK, p38 MAPK, and MAPK activation in ASM cells. Cultured ASM cells were treated for the indicated times with 200 ng/mL of TNF, 1 μM anisomycin, or 10 μM PDBu before analysis of kinase activity as described in the Methods section. The results shown are from a representative experiment from at least three other independent determinations with similar findings.

TNF treatment, suggesting a longer term, more secondary response. Treatment of the cells with the phorbol ester PDBu or the metabolic stressor anisomycin was used as positive control. TNF also has the ability to activate the ubiquitously expressed mitogenic MAPK pathway in most cell types. Unexpectedly, TNF here was only able to poorly activate this pathway in ASM cells (Fig. 3), with often no activation whatsoever. The inability of TNF to activate p42 and p44 MAPK has interesting consequences in relation to TNF signalling in ASM and to TNF's ability to activate hypercontractility in the muscle tissue.

One such signalling substrate molecule for activated MAPK is the hormone-sensitive lipase $cPLA_2$. $cPLA_2$ selectively hydrolyses arachidonic acid for the generation of eicosanoid inflammatory mediators and is involved in other systems in TNF-mediated actions, presumably through the cytokine's activation of MAPK pathways [26]. In ASM cells, TNF is unable to cause any marked activation of $cPLA_2$ enzyme. Fig. 4 shows that the phosphorylation (and activation) of $cPLA_2$ in ASM was unaffected by prolonged TNF treatments, despite PDBu's easily and rapidly causing the activation of the enzyme. In addition to $cPLA_2$ Western analyses, confocal immunocytochemical analysis of cultured ASM cells also revealed no alteration of $cPLA_2$ enzyme by TNF treatment (Fig. 4). In these experiments, TNF was unable to markedly alter the subcellular distribution and immunoreactivity of the lipase. $cPLA_2$ contains a C2 domain that is thought to confer Ca^{2+} sensitivity on the enzyme. Here, we observed within the same experiments that raising the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured ASM cells by Ca^{2+} ionophore (ionomycin) treatment led to the subcellular redistribution of $cPLA_2$. Other cell types analysed concurrently, such as HeLa, were responsive to both TNF- and Ca^{2+} ionophore-stimulated redistribution of $cPLA_2$ (Fig. 4 and [1]). It therefore appears that in ASM cells, TNF is largely unable to activate either the MAPK pathway or its $cPLA_2$ target.

Recent work has focused on the ability of TNF to activate the NFκB transcription factor that switches on a variety of NFκB-responsive genes crucial to mediating a range of inflammatory and immune responses [27,28]. By the use of transfection protocols and reporter construct technology, we investigated the ability of TNFR subtypes in cultured ASM cells to control this important transcriptional event. Over a period of 5 hr, TNF caused a substantial activation of the NFκB construct (Fig. 5). This indicates that any NFκB-responsive genes (such as those for interleukins) would be activated in ASM presented with TNF in an inflammatory condition. Moreover, agonistic TNFR subtype-specific antisera revealed that this activation of NFκB gene activity was mediated through the TNFR1 present on ASM cells, but not the TNFR2.

It is thought that activation of the stress kinase pathways may play a role in the onset of TNF-induced apoptotic cell death. Likewise, NFκB-responsive genes are thought to be able to stimulate the proliferation of a variety of cell types [14]. Using the crystal violet viable cell staining assay [22], we discovered that TNF was unable to alter cell death or proliferative processes in cultured ASM cells (Fig. 6). The TNF concentration–response relationship was unchanged even up to 200 ng/mL (11 nM) of TNF, a concentration of the cytokine that negates any possibility of underactivation of the TNFRs due to species differences or reduced activity of the cytokine protein. Concurrently run experiments on HeLa cells (a model which responds to TNF by undergoing apoptotic cell death) showed the expected activity of TNF in reducing cell number. In addition, 1 μM PDBu-treated HeLa cells proliferated in 24 hr to 134 ± 7% of control cell numbers ($P < 0.05$). Thus, it appears that TNF neither reduced nor increased ASM cell numbers, and was unable to elicit a cytotoxic or proliferative response in these cells, at least in the time periods measured here.

4. Discussion

Recent studies have increasingly focused on TNF as a mediator of airway hypersensitivity in diseased states such as spasmogen-induced asthma and chronic obstructive pulmonary disease. Due to the nature of muscle contractile responses, alteration of Ca^{2+}-sensing cellular machinery is the root cause of the increased ASM contraction seen under these clinical conditions. Thus, early investigations analysed the modulation by TNF of Ca^{2+} movements within cultured ASM cells [11,12,29–31]. This work uncovered modulations of inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} release and flux mechanisms. The times of these observations, however, were around 24 hr of TNF treatment, and given the more rapid onset of asthmatic hyperconstriction, a more timely modulation of contractile responses was sought. Our observations in whole and permeabilised tissue revealed that TNF can cause a far more rapid and profound modulation of the contractile response of ASM [13]: TNF treatments of 20–45 min caused up to a 4-fold greater response to contractile agonists such as carbachol and serotonin. TNF did not itself produce a contraction but sensitised the smooth muscle contractile response to other contractile agents. This sustained TNF effect was to cause a Ca^{2+} sensitisation of the smooth muscle myofilaments, ultimately leading to the enhanced phosphorylation of the myosin light chain 20 (MLC_{20}).

TNFRs are single transmembrane glycoproteins and are not of the classic seven-transmembrane G protein-coupled receptor superfamily that is responsible for rapid release of intracellular Ca^{2+} ions observed in fast contractile responses, although they do possess the ability to modulate pathways by which released Ca^{2+} may act. TNFRs affect the intracellular environment through the activation of a range of kinase pathways. Here, we show that TNF signif-

[1]McFarlane SM, Jupp OJ and MacEwan DJ, unpublished observations.

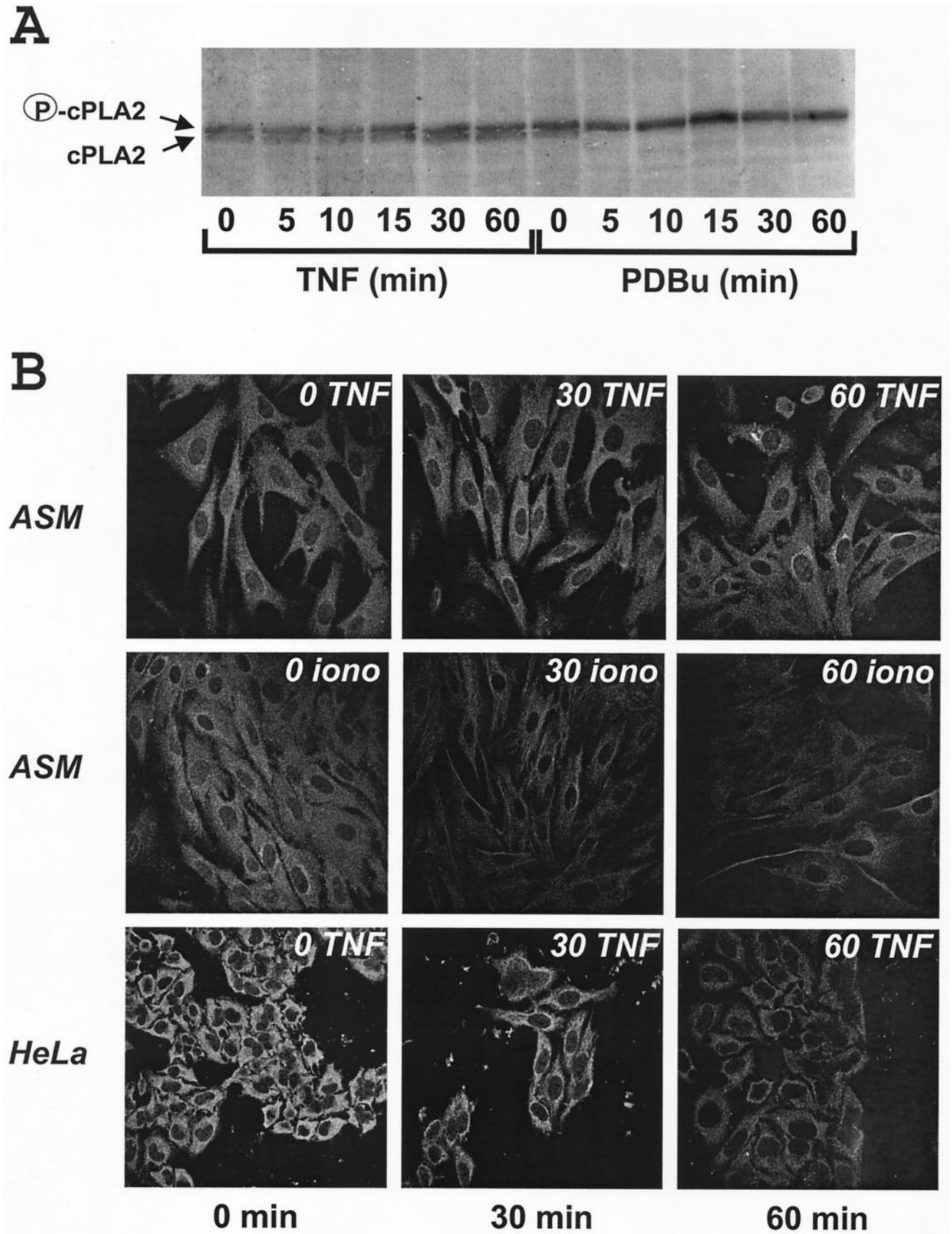

Fig. 4. Lack of TNF-induced $cPLA_2$ activation in ASM cells. (A) Cultured ASM cells were treated for the indicated times with 200 ng/mL of TNF or 10 μM PDBu before Western analysis of the $cPLA_2$ phosphorylation state as described in the Methods section. (B) Stimulus-induced subcellular redistribution of $cPLA_2$ in ASM and HeLa cells measured by laser confocal microscopy. Cells were treated for the indicated times with 200 ng/mL of TNF or 1 μM ionomycin (iono) as specified. $cPLA_2$ protein redistributes from cytosolic (non-nuclear) sites to more perinuclear locations. The results shown are from a representative experiment from at least two other independent determinations with similar findings.

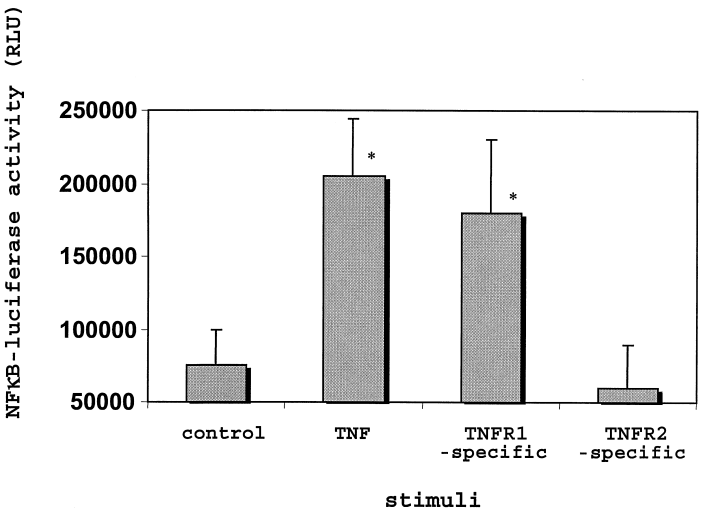

Fig. 5. TNF-stimulated NFκB activation in ASM. Cultured ASM cells were transfected with an NFκB–luciferase reporter construct as described in the Materials and Methods section. Forty-eight hours posttransfection, the cells were treated for 5 hr with 200 ng/mL of TNF or a 1:100 dilution of agonistic TNFR-specific antisera. The data represent the means ± SD of triplicate determinations with a representative experiment shown. Statistical significance from control values ($P < 0.05$, Student's *t*-test) are shown (*). RLU, relative light units.

icantly activated the JNK, p38 MAPK, and NFκB-inducing stress-activated kinases, but did not significantly activate p42 MAPK or p44 MAPK pathways. The inability of TNF to activate MAPK in ASM cells may be due to unforeseen experimental factors not investigated here. For example, cell metabolic activity and culture confluency may affect the ability of this kinase to be activated. Moreover, species differences in the TNF used and its cytokine activity may account for some of its inability to affect this particular kinase pathway. It cannot be overlooked that in guinea pig

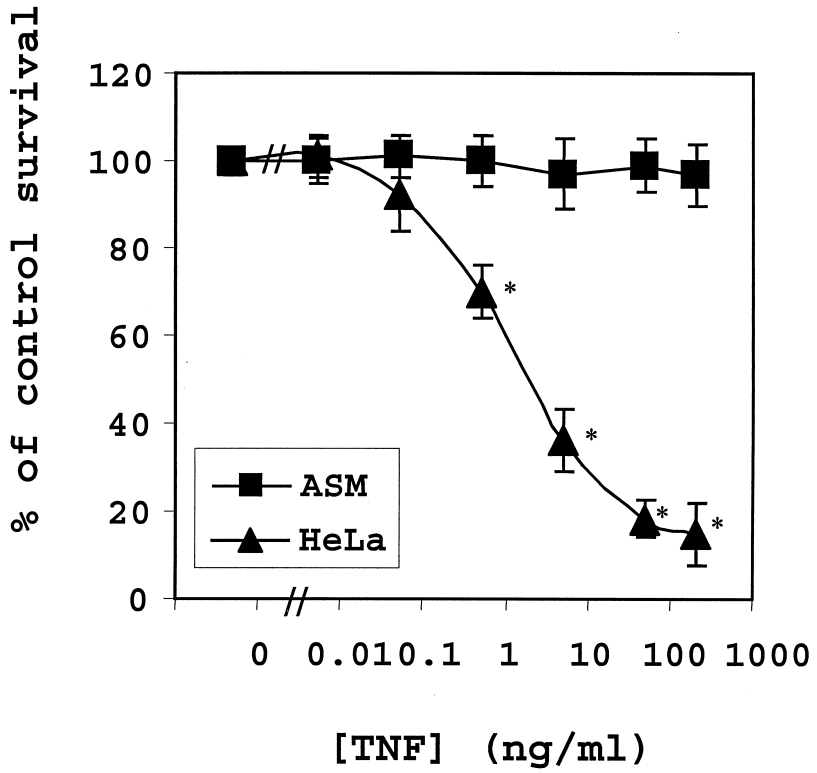

Fig. 6. TNF concentration-cytotoxicity/proliferation response measurements in ASM cells. Cultured ASM or HeLa cells were treated for 24 hr with the indicated concentrations of TNF before measurement of viable cell number as described in the Methods section. The data shown represent the means ± SD of eight determinations from a representative experiment from at least two other independent determinations with similar findings. Statistical significance from control values ($P < 0.05$, Student's *t*-test) are shown (*).

cells in this study, the same sample elicited p38 MAPK but not p42/p44 MAPK activation. Such a profile of TNF-activated kinases has not been reported in muscle tissue. It may be that MAPK is not efficiently activated by TNF receptors in such cell types, giving only a stressor response in these cells, which raises interesting questions regarding the TNFR/TRAF interactions in ASM that govern activation of these kinase pathways.

Further evidence for the inability of TNF to activate MAPK comes from our studies on the $cPLA_2$ enzyme. MAPK is thought to be the main enzyme capable of phosphorylating $cPLA_2$ to cause its greater activity [32]. However, others have reported that $cPLA_2$ may also be phosphorylated and activated by the p38 MAPK, JNK, and protein kinase C pathways [33–37]. Our evidence here supports a role in the phosphorylation and activation of $cPLA_2$ for MAPK, but not p38 MAPK or JNK. We observed significant p38 MAPK and JNK activities stimulated by TNF, but were unable to observe any alteration of $cPLA_2$ phosphorylation. The activation-induced subcellular redistribution of $cPLA_2$ is similar to that observed by others [38–40] in that activation of the enzyme (in part by raised Ca^{2+} levels), leads to its rapid movement from the cytosol to perinuclear regions. The observed reduction in immunofluorescence intensity of activated $cPLA_2$ may be due to a number of factors. A simple reduction in the diffuse distribution of the enzyme may give an apparent decrease in overall observable fluorescence intensity. Additionally, recent work has suggested that activated $cPLA_2$ undergoes rapid proteolytic cleavage by caspase enzymes [41,42]. This proteolytic cleavage of the active $cPLA_2$ enzyme may also contribute to the reduction in observed fluorescence. Moreover, the $cPLA_2$ antisera used in these studies [43,44] are distinct from the sera used in the work from other groups. The binding properties of this antibody may be altered after proteolytic cleavage of its $cPLA_2$ target.

It is interesting that $cPLA_2$ does not appear to be involved in TNF-stimulated actions in ASM cells. $cPLA_2$ is the rate-limiting step in the generation of prostaglandin and leukotriene inflammatory mediators. It is known that leukotrienes can influence basal contractile tone of ASM. It may be that TNF-stimulated hyperreactivity of ASM does not depend on eicosanoid generation, but more on a stress kinase response such as with JNK, p38 MAPK, or NFκB activities as uncovered here. $cPLA_2$ is thought by some to be involved in regulating cell cycle, proliferative/apoptotic processes. Our results do not preclude such a role, as not only was TNF unable to activate $cPLA_2$ actions, but it did not alter ASM proliferative or apoptotic responses. Likewise, significant activation of the NFκB transcription factor by TNFR1 was observed in ASM cells, a step thought to be important in the proliferative responses of TNF [45], although TNF did not alter ASM cell proliferation over long-term (>24 hr) treatment. The profile of kinase activation uncovered here may indicate that TNF is more important in short-term stressing of the muscle. It is not clear if the TNF-stimulated p38 MAPK and JNK cascades result in NFκB-mediated gene induction, although it is more likely that a distinct TNF-stimulated kinase pathway is responsible for the activation of transcription factor. NFκB activity has been shown to be involved in the induction of intercellular adhesion molecule-1 and CD40 in ASM cells in response to TNF [29,31]. Interestingly, TNF-stimulated CD40 production led to CD40-mediated regulation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), which may contribute to short-term TNF-induced hyperresponsiveness in ASM. The time scale of TNF-stimulated Ca^{2+} sensitisation would require rapid cellular effects (such as kinases), and NFκB-stimulated gene activity may be more crucial in longer-term effects in ASM cells, although *in vivo* the TNF-stimulated signalling machinery may be optimally organised to elicit such rapid physiological effects. Clearly, more work needs to be undertaken to delineate the precise molecular role of TNF and its receptors in airway smooth muscle.

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