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Activation of p55 Tumor Necrosis Factor-α Receptor-1 Coupled to Tumor Necrosis Factor Receptor-Associated Factor 2 Stimulates Intercellular Adhesion Molecule-1 Expression by Modulating a Thapsigargin-Sensitive Pathway in Human Tracheal Smooth Muscle Cells

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ABSTRACT

Tumor necrosis factor-α (TNFα) stimulates the expression of intercellular adhesion molecule-1 (ICAM-1) by activating the transcription factor nuclear factor-κB (NF-κB) in human airway smooth muscle (ASM) cells. This study characterizes the receptor involved as well as critical downstream signaling events mediating cytokine-induced NF-κB activation and ICAM-1 expression. TNFα stimulation for 1 to 4 h induced ICAM-1 expression in human ASM cells. This rapid TNFα-induced ICAM-1 expression enhanced T-lymphocyte adhesion to ASM cells, which was inhibited by anti-ICAM-1 antibodies. Using immunostaining, we demonstrated that TNFα receptors TNFR1 and TNFR2 are expressed on native human tracheal smooth muscle. Treatment of cells with htr-9, an antibody that specifically activates TNFR1, also stimulated expression of ICAM-1 mRNA and protein. Utr-1, a blocking antibody to TNFR2, did not affect TNFα-mediated ICAM-1 expression. Both TNFα and htr-9 increased luciferase activity in ASM cells transfected with a NF-κB reporter plasmid. Overexpression of a dominant negative TNF receptor-associated factor 2 construct, lacking the NH_2-terminal RING finger, completely abrogated both TNFα- and htr-9-mediated increases in NF-κB reporter activity. Thapsigargin, an agent that depletes intracellular calcium stores, abrogated both cytokine-mediated NF-κB-dependent ICAM-1 mRNA transcription and protein expression but had no effect on IκB degradation. In addition, chelating cytosolic calcium with 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester also inhibited cytokine TNFα-induced ICAM-1 expression. These data suggest that TNFR1, through a TNF receptor-associated factor 2-NF-κB signaling pathway, mediates TNFα-induced expression of ICAM-1 on ASM cells by involving a thapsigargin-sensitive signaling pathway.

Smooth muscle plays a central role in the pathogenesis of a variety of diseases, including atherosclerosis and asthma. Although the primary function of smooth muscle was thought to regulate vascular and airway resistance through contraction, recent evidence suggests that smooth muscle has other important functions in health and disease. The synthetic function of smooth muscle, which includes cytokine and growth factor secretion and cell adhesion molecule expression, may serve to orchestrate and perpetuate local inflammatory responses (Libby and Hansson, 1991).

In vitro studies performed with cultured cells have allowed us and others to pinpoint many factors that can lead to modification of airway smooth muscle (ASM) cell function. In this regard, cytokines, important mediators of inflammation, have been shown to directly modulate ASM cell responsiveness (for review, see Amrani and Panettieri, 1998). In addition, tumor necrosis factor-α (TNFα) and interleukin-1β (IL1β) stimulate intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and CD44 that enhance adhesion of activated T cells to ASM cells (Lazaar et al., 1994; Panettieri et al., 1995). New evidence suggests

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ABBREVIATIONS: ASM, airway smooth muscle; TNFα, tumor necrosis factor-α; IL1β, interleukin-1β; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; NF-κB, nuclear factor-κB; TRADD, TNF receptor-associated death domain; TNFR, TNF receptor; TRAF2, TNF receptor-associated factor 2; DN, dominant negative; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; CHX, cycloheximide; IκB, inhibitor of κB; RANTES, regulated upon activation normal T cell expressed and secreted.

that TNFα and IL1β stimulate the secretion of IL8, IL6, RANTES, and granulocyte-macrophage colony-stimulating factor (for review, see Johnson and Knox, 1997). Although cytokines, which activate specific cell surface receptors, modulate smooth muscle cell synthetic responses, the downstream signaling mechanisms by which cytokines mediate these effects remain unclear.

In smooth muscle, compelling evidence suggests that cytokines mediate some of their cellular effects by activation of the transcription factor nuclear factor-κB (NF-κB) (for review, see Johnson and Knox, 1997). Our laboratory has recently shown that TNFα and IL1β induce ICAM-1 expression in an NF-κB-dependent manner (Amrani et al., 1999). However, the signaling events in smooth muscle that couple cytokine receptors to activation of NF-κB remain unknown. Evidence in other cell types demonstrated that the upstream signals that activate NF-κB include intracellular signal proteins termed TNF receptor-associated death domain (TRADD; Hsu et al., 1995). Upon engagement of TNFα receptor-associated factor-1 (TNFR1), TRADD acts as an adapter by recruiting the downstream transducer TNF receptor-associated factor 2 (TRAF2), which mediates NF-κB activation (Hsu et al., 1995, 1996). In other cell types, however, a TRAF2-independent activation of NF-κB by TNFα also has been described (Lee et al., 1997). Furthermore, TRAF2 can interact with TNFR2 directly to activate NF-κB in some cell types (Rothe et al., 1994). These studies suggest that the activation of TRAF2 by TNFR subtypes is complex and may be cell specific. The role of TRAF proteins in human smooth muscle cells has not been investigated. In the light of the pivotal role of NF-κB in the regulation of proinflammatory genes in ASM cells, the identification of the TNFα signal transduction mechanisms that regulate NF-κB activation is potentially of therapeutic interest.

Evidence also suggests that an increase in cytosolic calcium is an important second messenger that modulates cytokine-induced NF-κB activation. Agonists that mobilize calcium from internal stores (Pahl and Baeuerle, 1996) or from extracellular sources (Kanno and Siebenlist, 1996) are able to induce NF-κB activation in HeLa cells and T cells, respectively. In human ASM cells, we recently reported that nickel, which prevents calcium influx, abrogated CD40-induced NF-κB activation (Lazaar et al., 1998), showing a physiologically relevant interaction between cytosolic calcium fluxes and activation of NF-κB in human ASM cells. However, the precise role of intracellular calcium on cytokine-induced NF-κB activation as well as NF-κB-dependent gene transcription remains unknown.

In this study, we show that direct engagement of TNFR1 with the activating antibody htr-9 stimulates a rapid expression of ICAM-1 on human ASM cells and that TNFα-induced ICAM expression promotes adhesion of T lymphocytes. Htr-9 also activates expression of an NF-κB-dependent luciferase reporter gene. Cotransfection of ASM cells with a dominant negative (DN) construct of TRAF2 abrogates this response. Finally, we demonstrate that thapsigargin, a potent sarcoendoplasmic calcium-ATPase inhibitor, suppresses TNFα-mediated NF-κB-dependent transcription as well as ICAM-1 protein expression. 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA-AM), an intracellular calcium chelator, also abrogates TNFα-mediated ICAM-1 expression. Together, these data suggest that TNFR1 coupled to TRAF2 plays a central role in NF-κB-mediated gene expression in ASM cells, and that thapsigargin-sensitive calcium pools modulate TNFα-induced NF-κB activation and ICAM-1 expression.

Materials and Methods

ASM Cell Culture. Human trachea was obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was removed under sterile conditions and the trachealis muscle isolated. With this technique, ~0.5 g of wet tissue was obtained, minced, centrifuged, and resuspended in 10 ml of buffer containing 0.2 mM $CaCl_2$, 640 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 10 U/ml elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 105-μm Nytex mesh, and the filtrate was washed with equal volumes of cold Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Aliquots of the cell suspension were plated at a density of 1.0×10^4 cells/cm^2. The cells were cultured in Ham's F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml amphotericin B and this medium was replaced every 72 h. Cell counts were obtained from triplicate wells with a 0.5% trypsin in 1 mM EDTA solution.

Human ASM cells in subculture during the second through to fifth cell passages were used because, during these cell passages, the cells retain native contractile protein expression, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin (Panettieri et al., 1989). These cells retain functional cell-excitation coupling systems as determined by Fura-2 measurements of agonist-induced changes in cytosolic calcium (Panettieri et al., 1989). All experiments were performed with a minimum of three different cell lines. Each ASM cell line was established with tracheal tissue from a single human donor.

Immunostaining of TNFα Receptors on Human Tracheal Sections. Sections of human tracheal smooth muscle were washed with HEPES buffer containing 137.5 mM NaCl, 1.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 6 mM KCl, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA (wt/vol). The tissues were fixed with 4% paraformaldehyde solution for 30 min at room temperature, then washed three times with HEPES buffer. The tissues were then permeabilized with cold methanol (−20°C), washed three times with HEPES buffer, and incubated with mouse anti-TNFR1 (htr-9) and anti-TNFR2 (utr-1) antibodies (provided by Dr. W. Lesslauer, Hoffman La Roche, Basel, Switzerland) for 120 min at 25°C. Negative controls included tissues incubated in the absence of the primary antibody. Immunoperoxidase staining was performed with the Vectastain kit and DAB substrate kit (Vector Laboratories, Burlingame, CA). After washing, the glass coverslips were mounted onto glass slides and examined under epifluorescence microscopy (Nikon, Tokyo, Japan) and photographed.

Flow Cytometry. Flow cytometric analysis was performed as described previously (Lazaar et al., 1994; Amrani et al., 1999). Human ASM cells were stained with either a fluorescein isothiocyante-conjugated monoclonal antibody specific for ICAM-1 or an isotype matched control (R&D Systems, Minneapolis, MN). Samples were then analyzed with an EPICS XL flow cytometer (Coulter Corporation, Hialeah, FL). ICAM-1 expression is presented as the increase in mean fluorescence intensity over background.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis. ASM cells were washed with cold PBS and resuspended in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 10 μg/ml aprotinin and leupeptin. The cell lysate was kept on ice for 20 min and clarified by centrifu-

gation at 12,000 rpm for 5 min. Proteins present in the supernatant were analyzed on a 12.5% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. The membranes were blocked in 3% BSA in Tris-buffered saline, then incubated with a rabbit polyclonal IgG anti-TRAF2 or rabbit anti-IκBα antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the peroxidase-conjugated secondary antibody (Boehringer Mannheim, Minneapolis, MN) at room temperature in the same buffer, the bands were visualized by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Immunostaining of NF-κB in Cultures of Human ASM. Cultures of ASM were maintained in serum-free medium supplemented with insulin (5.7 mg/ml) and transferrin (5 mg/ml) for 48 h before the addition of TNFα (10 ng/ml) for 60 min. Some cultures were pretreated with thapsigargin (10 nM) or vehicle control (0.0001% v/v dimethyl sulfoxide) for 5 min before the addition of TNFα. Cultures were rinsed three times with PBS, then fixed for 10 min in 4% formaldehyde in PBS. After fixation, cells were rinsed three times with PBS, then permeabilized in 0.5% Triton X-100 in PBS for 20 min. Cells were rinsed three times with PBS and blocked with 1% BSA in PBS for 30 min. Cells were incubated with primary antibody anti-p65 NF-κB, 1 μg/ml in 0.25% BSA in PBS (Santa Cruz Biotechnology) for 60 min at 37°C, then rinsed three times with PBS. Cells were then incubated with 2 μg/ml secondary antibody, goat anti-rabbit conjugated to biotin (Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature, then rinsed three times with PBS and incubated with 2 μg/ml streptavidin-Texas Red (Jackson ImmunoResearch) for 30 min at room temperature. Cells were then rinsed three times with PBS and the coverslips were mounted in 80% glycerol in PBS. Immunostained cells were visualized on an Olympus IX 710 fluorescence microscope.

Quantitative Adhesion Assay. T-lymphocyte adhesion was performed as described previously (Lazaar et al., 1994). T lymphocytes were isolated from normal volunteers with Ficoll gradient centrifugation and E-rosetting with neuraminidase-treated sheep erythrocytes. Cells were stimulated with phorbol-12,13-dibutyrate and ionomycin and labeled with 2 μCi/ml ^{3}H-labeled thymidine (40–60 mmol; DuPont NEN, Boston, MA) during the last 12 to 18 h of culture. Unstimulated or activated T cells were added to ASM cells that had been pretreated with 10 ng/ml TNFα for 4 h. After 1 h at 37°C, nonadherent T cells were removed by washing. Adherent T cells were lysed with 1% Triton X-100 in PBS and counted with a beta counter. Each condition was performed in triplicate and data are expressed as the mean of percentage of input cells bound ± S.D. For blocking antibody studies, T cells and ASM cells were incubated for 30 min with 10 μg/ml mouse anti-human ICAM-1 (RR6.5, kind gift of R. Rothlein, Boehringer Ingleheim, Ridgefield, CT; Rothlein et al., 1988).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. RT-PCR for ICAM-1 was carried out as reported previously (Amin et al., 1995). Briefly, cells were homogenized in 4 M acid guanidinium thiocyanate, and phenol-chloroform extracted and ethanol precipitated to recover total RNA. The ICAM-1 primers for PCR analysis were 5′-CTTCTCCTGCTCTGCAACCC-3′ (base 1104–1123, sense) and 5′-GGGAGAGCACATTCACGGTC-3′ (base 1429–1410, antisense; Satoh et al., 1994). Each of 28 cycles of the PCR was programmed to carry out denaturation at 94°C for 60 s, primers annealing at 60°C for 45 s, extension at 72°C for 2 min, and a final extension at 72°C for 8 min. The semiquantitative PCR approach of ICAM-1 mRNA was performed in parallel by investigating human β-actin mRNA levels with the following primers: 5′-ATGGATGATGATATCGCCGC-3′ (sense) and 5′-TTAATGTCACGCACGATTTC-3′ (antisense) as described in Amin et al. (1995).

Transfection of Human ASM Cells. Transfection of human ASM cells was performed as described previously (Amrani et al., 1999). Briefly, ASM cells were transfected with 2 μg of pNF-κB-Luc designed for monitoring activation of NF-κB (Clontech, Palo Alto, CA) and 2 μg of pSV-β-galactosidase control vector to normalize transfection efficiencies (Promega, Madison, WI). Cells also were tranfected with 1 μg of pRK-TRAF2 (87–501; kind gift from Dr. D. Goeddel, Tularik, Inc., South San Francisco, CA), which acts as DN of TRAF2, or with empty vector. Forty-eight hours after transfection, cells were quiesced in medium containing 0.2% FBS for 16 h and exposed to 10 ng/ml TNFα or 30 μg/ml htr-9 for 4 h. Cells were then harvested and luciferase and β-galactosidase activities were assessed with a Promega kit according to the manufacturer's instructions.

Statistical Analysis. One-way ANOVA was used on all data when experiments were of a factorial design. Fisher's protected least-significant difference multiple comparison test was used to compare differences between treatment means. Correlations between two variables were performed with linear regression analysis. For all analyses, effects were considered statistically significant if the probability (P) of the effect being due to chance alone was <5%.

Results

TNFα Increases ICAM-1 Expression, Which Mediates Binding of Activated T Lymphocytes to ASM Cells. We examined the early time course of ICAM-1 expression on human ASM cells after stimulation with TNFα. TNFα (10 ng/ml) treatment of cells for 1 to 4 h induced a time-dependent increase in ICAM-1 expression (Fig. 1A). The net fold increases in ICAM-1 protein over basal after 1, 2, 3, and 4 h of incubation were 1.3 ± 0.2, 1.6 ± 0.1, 2.9 ± 0.3, and 6.2 ± 0.4 for TNFα, respectively (significantly different from control as early as 2 h; $n = 4$). The dose of TNFα used in these experiments was chosen because our previous studies show that this concentration maximally induced ICAM-1 expression (Lazaar et al., 1994). To test whether rapid expression of ICAM-1 was due to protein synthesis, human ASM cells were stimulated with TNFα in the presence or absence of 10 μM cycloheximide (CHX). CHX completely prevented cytokine-mediated increase in ICAM-1 expression (Fig. 1A; $^{\#}P < .001$, $n = 4$), suggesting that de novo protein synthesis was required for the TNFα-mediated CAM expression.

We previously showed that ICAM-1 expression induced by cytokines plays an important role in T-lymphocyte adhesion to human ASM cells (Lazaar et al., 1994). However, T-cell adhesion in ASM cells exposed to cytokines for short incubation times, when ICAM-1 but not VCAM-1 is expressed, was not studied. We investigated whether cytokine-mediated increases in T-lymphocyte adhesion at 4 h were ICAM-1-dependent. Using an in vitro adhesion assay, we show that resting T cells adhered minimally to unstimulated ASM cells, and that adhesion was not increased by pretreating ASM cells with TNFα (Fig. 1B). In contrast, 31% of activated T cells adhered to unstimulated ASM cells. This increased to 69% after treatment with TNFα for 4 h. T-cell adhesion to human ASM cells was completely blocked when cells were preincubated with blocking antibodies specific for ICAM-1 as shown in Fig. 1B. These data suggest that the TNF-inducible adhesion of activated T lymphocytes is mediated solely by the rapid expression of ICAM-1.

TNFR1 Engagement Stimulates a Rapid Increase in ICAM-1 mRNA and Protein in Human ASM Cells. We have previously shown that htr-9, a specific agonist antibody to TNFR1, can mimic the effects of TNFα on ASM cells by potentiating agonist-evoked calcium transients and by stimulating mitogenesis (Amrani et al., 1996). To investigate whether TNFR1 also increases total ICAM-1 mRNA, we

studied the effect of htr-9 on ICAM-1 mRNA levels. Incubation of ASM cells with htr-9 (30 μg/ml) increased ICAM-1 total mRNA expression in a time-dependent manner (1 to 4 h; Fig. 2A). Semiquantitative analysis of htr-9-induced ICAM-1 mRNA expression (Fig. 2B) was investigated by calculating the ratio to the concomitant expression of human β-actin mRNA, a gene constitutively expressed in ASM cells. The net increases in htr-9-induced ICAM-1 mRNA were time-dependent with densitometric values of 0.18, 0.30, 0.35, and 0.40 at 1, 2, 3, and 4 h, respectively, after treatment with htr-9 (Fig. 2B). Similar results were obtained in mRNA expression when cells were stimulated with TNFα (data not shown). This provides evidence that TNFR1 activation induces ICAM-1 gene expression.

To determine whether TNFR1-induced increases in mRNA levels correlated with cell surface ICAM-1 expression, cells were treated with htr-9 for 4 h and ICAM-1 expression was then measured by flow cytometry. The htr-9 (30 μg/ml) antibody stimulated a 3.2 $\pm$ 0.1-fold increase in ICAM-1 expression over cells treated with diluent alone ($P < .01$, $n = 3$; Fig. 3A). By 24 h, ICAM-1 expression in response to htr-9 increased 19 $\pm$ 0.3-fold (data not shown). No effect was observed in cells treated with utr-1 alone, an antibody with antagonistic properties against TNFR2p75. In addition, we found that pretreatment with utr-1 did not affect TNFα-induced ICAM-1 expression at maximal and submaximal doses (data not shown), which suggests that TNFR2 does not modulate the ICAM-1 expression in human ASM cells. Together, these data indicate that TNFR1 activation regulates the early increase in ICAM-1 induced by TNFα.

To rule out the possibility that the inability of utr-1 to block TNF-induced ICAM-1 expression was due to the absence of TNFR2, we stained freshly obtained tracheal smooth muscle with antibodies specific for TNFR1 and TNFR2. As demonstrated in Fig. 3B, both receptors are expressed on human ASM cells. This agrees with our previous studies

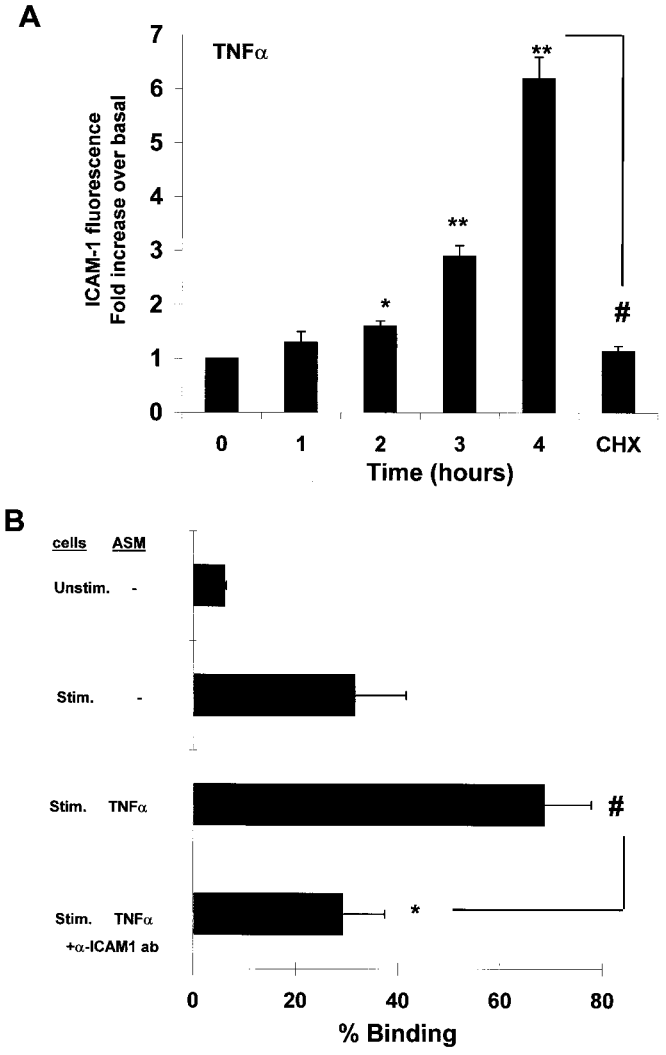


Fig. 1. Cytokine-induced rapid ICAM-1 expression on human ASM promotes adhesion of activated T lymphocytes. A, ASM cells were incubated for the indicated time with 10 ng/ml TNFα. In other experiments, cells were pretreated with CHX for 30 min and then stimulated with TNFα for 4 h. ICAM-1 expression was analyzed by flow cytometry as described in *Materials and Methods*. Values shown are mean $\pm$ S.E. ($n = 4$ separate experiments), $^{*}P < .05$, $^{**}P < .01$ compared with untreated cells, and $^{\#}P < .01$ compared with cells treated with TNFα. B, T cells were cultured without (unstim.) or with phorbol-12,13-dibutyrate and ionomycin (stim.) and pulsed with ^{3}H-labeled thymidine (1 μCi/10^{6} cells) for 16 h. ASM cells were pretreated with isotype matched antibodies in control media (unstim.) or with anti-ICAM-1 antibodies (10 μg/ml) for 45 min and then stimulated with TNFα for an additional 4 h. Adhesion assays were performed as described in *Materials and Methods*. Each condition was performed in triplicate ($n = 3$; $^{\#}P < .01$ compared with untreated ASM cells, and $^{*}P < .05$ compared with ASM cells treated with TNFα).

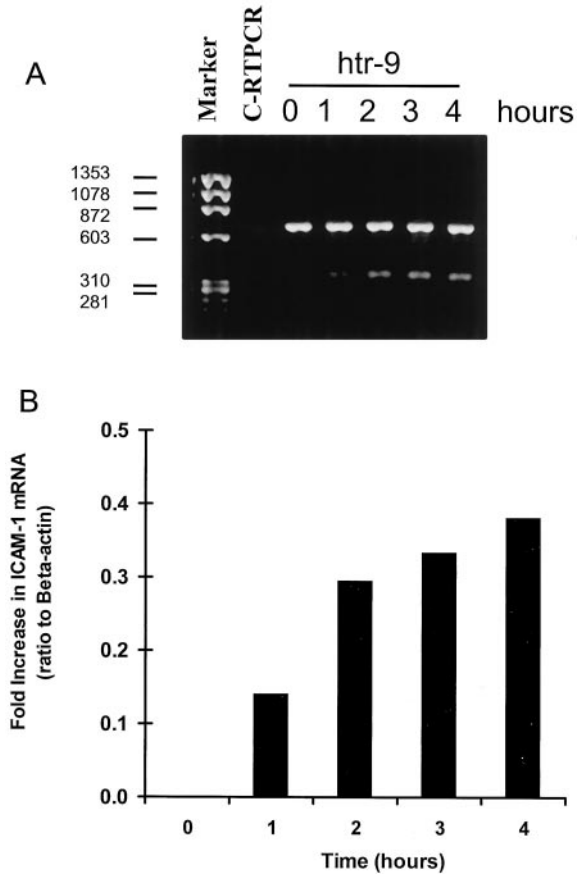
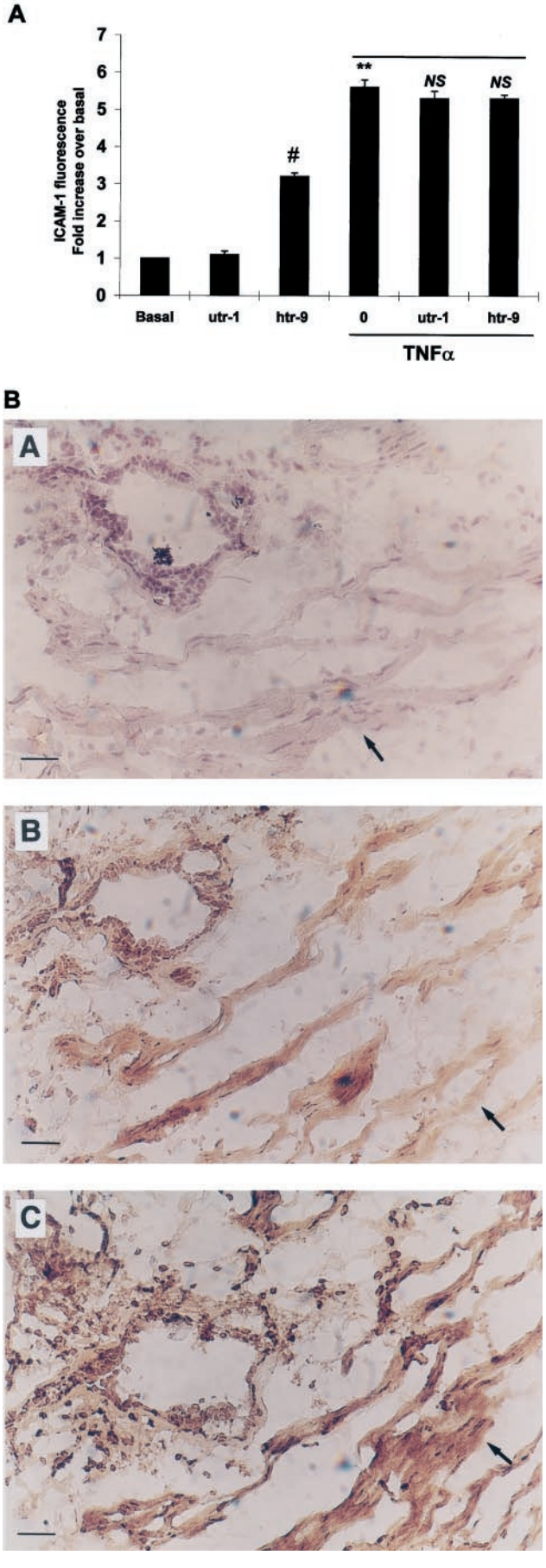


Fig. 2. Htr-9 stimulates ICAM-1 gene expression in human ASM cells. A, ASM cells were stimulated with 30 μg/ml htr-9 for the indicated times. Five micrograms of total RNA was subjected to RT-PCR with the primers for ICAM-1 (lower band) and β-actin (upper band) as described in *Materials and Methods*. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. B, scanning densitometry of gel. Data are representative of four separate experiments.



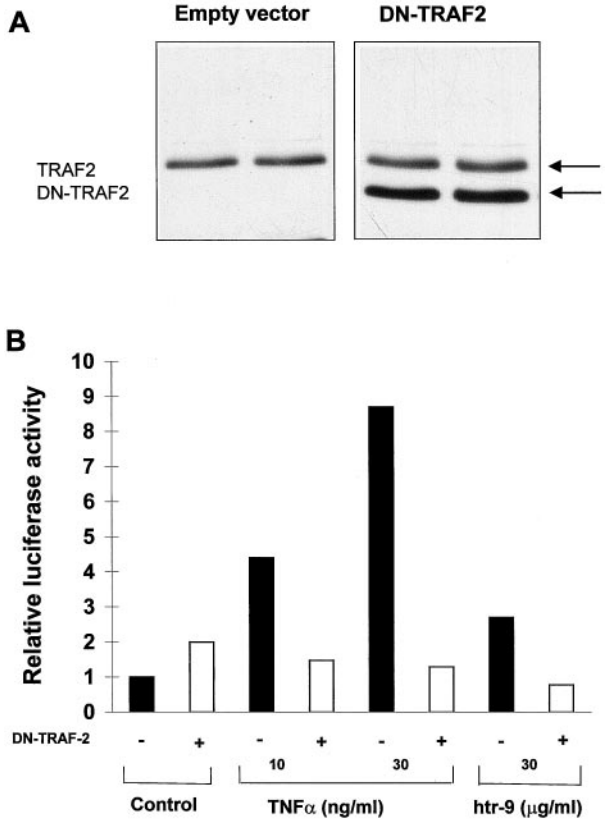


Fig. 4. Expression of dominant negative TRAF2 abrogates TNFα and htr-9-mediated NF-κB-luciferase reporter gene expression in human ASM cells. A, ASM cells were transfected with 1 μg of DN-TRAF2 DNA or 1 μg of empty vector, placed in media containing 0.2% serum for 16 h. Expression of TRAF2 and DN-TRAF2 in cytoplasmic extracts was then analyzed by immunoblotting. B, cells were cotransfected with 2 μg of NF-κB-luciferase reporter construct, 1 μg of DN-TRAF2 DNA, or empty vector. Cells were stimulated with TNFα or htr-9 for 4 h. Luciferase activity in cell extracts was normalized for β-galactosidase activity as described in *Materials and Methods*. Data represent normalized luciferase activity relative to untreated cells and are representative of three similar experiments.

demonstrating that TNFR1 and TNFR2 expression is maintained in cells cultured in vitro (Amrani et al., 1996).

TNFR1 Activation Stimulates NF-κB-Dependent Reporter Gene Activity in a TRAF2-Dependent Manner. Overexpression of a DN form of TRAF2, which lacks the NH_2-terminal RING finger (DN-TRAF2), has been shown previously to abrogate TNFα-induced NF-κB activation in some cell types (Hsu et al., 1996). To address whether TNF mediates NF-κB activation via TRAF2 in human ASM cells, we overexpressed DN-TRAF2 in ASM cells and measured NF-κB reporter gene activity in cells stimulated with TNFα.

Fig. 3. TNFR1 and TNFR2 are expressed on native human ASM but activation of TNFR1 mediates ICAM-1 expression in human ASM cells. A, cells were incubated with 30 μg/ml htr-9 or 30 μg/ml utr-1 (anti-TNFRp75) in the presence or absence of TNFα (10 ng/ml) for 4 h. ICAM-1 expression was assessed as described in *Materials and Methods*. Values shown are mean $\pm$ S.E. (n = 6 separate experiments). $^{**}P < .01$ and $^{\#}P < .01$ compared with untreated cells; NS not significant from cells treated with cytokine alone. B, sections of fresh tracheal tissues were incubated with no primary antibody (A), 10 μg/ml antibody against TNFR2 (B) or TNFR1 (C). Cell surface expression of TNFα receptors was assessed as described in *Materials and Methods*. Arrow locates the smooth muscle positively stained for TNFα receptors. This is a representative photomicrograph of tracheal sections from three donors showing identical patterns of TNFR expression.

As shown in Fig. 4A, overexpression of DN-TRAF2 does not alter the expression of endogenous TRAF2. We also found that ASM cells transfected with NF-κB-luciferase construct and control vector responded to increasing concentrations of TNFα with a dose-dependent increase in NF-κB reporter activity after 4 h (Fig. 4B). In addition, htr-9 (30 μg/ml), the activating antibody against TNFR1, also induced a 3-fold increase in NF-κB reporter activity. However, in ASM cells cotransfected with κB-luciferase construct and DN-TRAF2 vector, TNFα as well as htr-9 mediated NF-κB activation were completely abrogated. The response to TNFα was unaffected in cells transfected with vector alone. These results suggest that TNFR1 activation of NF-κB is TRAF2-dependent.

Thapsigargin Inhibits NF-κB-Dependent Transcription Induced by TNFα. Thapsigargin-sensitive calcium stores are thought to play a key role in the modulation of ASM cell function by cytokines (for review, see Amrani and Panettieri, 1998). In some cell types, evidence suggests that calcium originating from these stores plays a critical role in regulating NF-κB activation (Pahl and Baeuerle, 1996). We examined whether thapsigargin-sensitive calcium pools modulated TNFα-induced NF-κB activation and ICAM-1 expression. In ASM cells pretreated with thapsigargin, we found that thapsigargin, at 10^{-9} to 10^{-7} M, partially inhibited TNFα-induced ICAM-1 expression (52 ± 5% compared with cells treated with diluent; Fig. 5A).

To confirm that cytosolic calcium levels modulate TNFα-stimulated ICAM-1 expression, we performed experiments with the membrane-permeable calcium chelator BAPTA-AM (Tsien, 1980). BAPTA-AM has been recently used in different cell lines to characterize the role of intracellular calcium in the regulation of a variety of cellular responses (Chen et al., 1999; Takahashi et al., 1999; Terry et al., 1999; Xu et al., 1999). Cells were pretreated with BAPTA-AM and then stimulated with TNFα. ICAM-1 expression was then measured. As shown in Fig. 5B, BAPTA-AM caused a dose-dependent attenuation of the expression of ICAM-1 induced by TNFα, whereas it had no effect on the basal ICAM-1 expression. These data further support our results suggesting that cytosolic calcium levels modulate TNFα-induced ICAM-1 in human ASM cells. We also observed that at the same concentrations, thapsigargin blocked NF-κB-dependent luciferase activity induced by TNFα (Fig. 6A). Surprisingly, thapsigargin did not alter IκBα degradation (Fig. 6B) or NF-κB nuclear translocation (Fig. 7) induced by TNFα, suggesting that the suppressive effect of thapsigargin on NF-κB-dependent transcription occurs downstream, possibly at the level of DNA binding. Because we recently showed that NF-κB played an important role in cytokine-mediated ICAM-1 expression

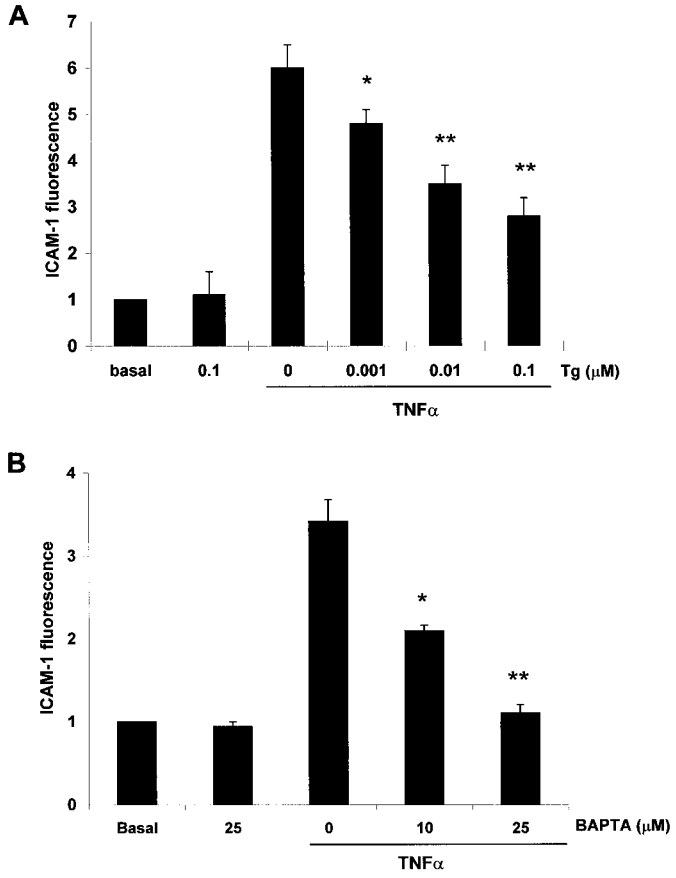


Fig. 5. Thapsigargin and BAPTA-AM inhibit TNFα-mediated ICAM-1 expression. ASM cells were preincubated with varying concentrations of thapsigargin for 5 min (A) or BAPTA-AM for 45 min (B) and then stimulated with 10 ng/ml TNFα for 4 h (A) and 3 h (B). ICAM-1 expression was analyzed by flow cytometry as described in *Materials and Methods*. Data represent mean ± S.E. (n = 3 separate experiments with each condition performed in duplicate; $^*P < .05$, $^{**}P < .01$ compared with cells treated with cytokine alone).

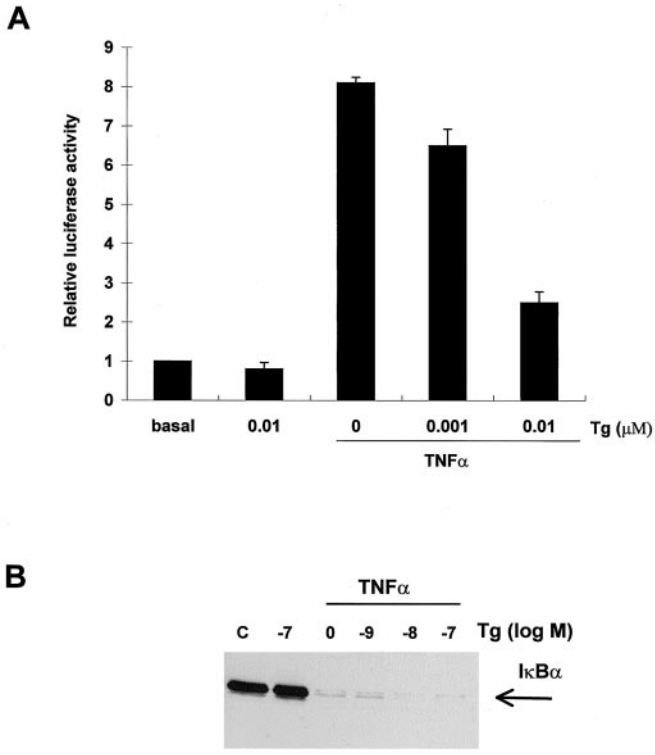


Fig. 6. Cytokine-mediated NF-κB-dependent gene transcription, but not IκBα degradation, is affected by thapsigargin. A, ASM cells transfected with the κB-luciferase reporter construct were pretreated with thapsigargin at the indicated concentration and then stimulated with 10 ng/ml TNFα for 4 h. Luciferase activity in cell extracts was normalized for β-gal activity as described previously (Amrani et al., 1999). Data represent normalized activities relative to the same cells without cytokine treatment and are representative of three similar experiments. B, cells were preincubated with the indicated concentration of thapsigargin were then stimulated with 10 ng/ml TNFα for 15 min before assessing IκBα degradation as described in *Materials and Methods* (n = 3).

(Amrani et al., 1999), these data suggest that thapsigargin modulates expression of ICAM-1 by suppressing NF-κB-dependent gene activation. The thapsigargin effects on ICAM-1 expression were not due to a nonspecific inhibition of protein transcription because β-actin mRNA expression was unaffected by thapsigargin pretreatment (data not shown).

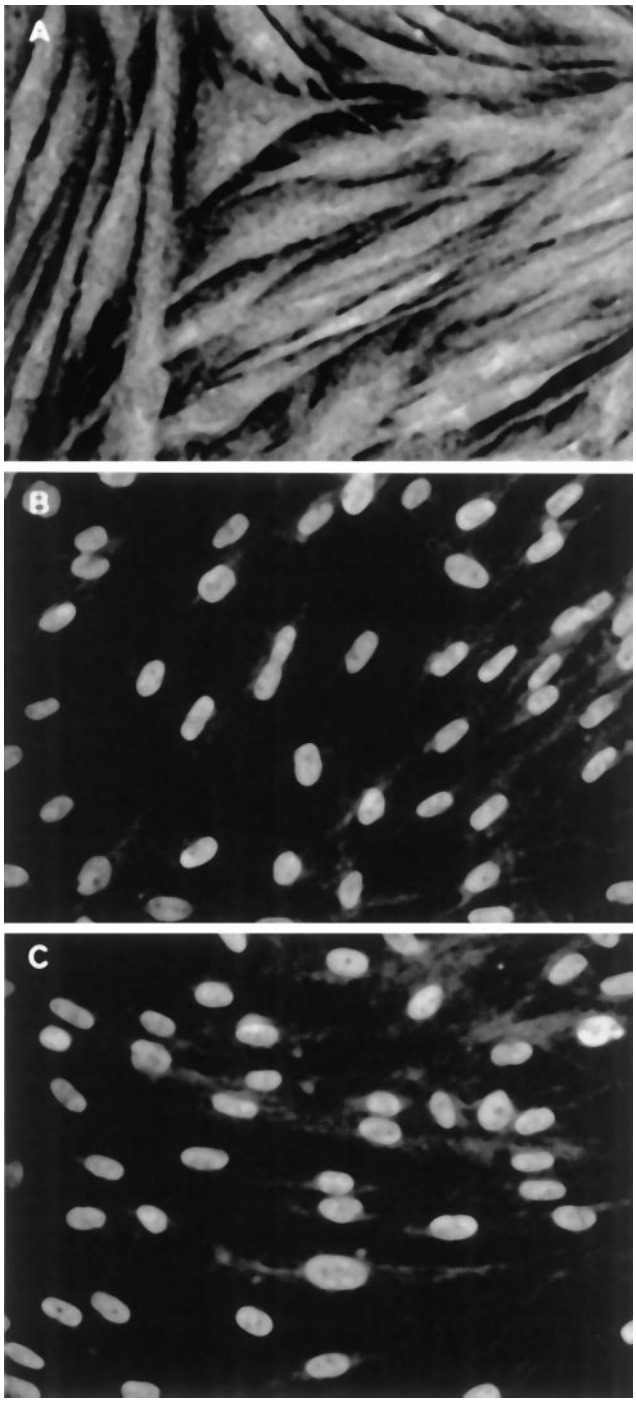


Fig. 7. Thapsigargin does not affect NF-κB nuclear translocation induced by TNFα in human ASM cells. Control cells (A) and cells stimulated with 10 ng/ml TNFα for 60 min in the absence (B) or the presence (C) of 0.01 μM thapsigargin added 5 min before stimulation. NF-κB nuclear translocation was assessed as described in *Materials and Methods*.

Discussion

We have extended our previous studies of cytokine effects on ASM cells by demonstrating that TNFα, via the activation of TNFR1 coupled to TRAF2, induces a rapid expression of cell surface ICAM-1 that, in turn, mediates adhesion of activated T lymphocytes to ASM cells. In addition, we found that both TNFα-induced NF-κB-dependent transcription and ICAM-1 expression were suppressed by thapsigargin, an agent that depletes internal calcium stores, or by BAPTA-AM, an agent that chelates intracellular calcium.

Previously, our laboratory reported that engagement of TNFR1 on ASM cells modulated the cellular response to contractile agonists (Amrani et al., 1996). With the htr-9 monoclonal antibody with agonistic activity on TNFR1 (Brockhaus et al., 1990; Shalaby et al., 1990), our data now suggest that activation of TNFR1 modulates expression of ICAM-1 on ASM cells. In addition, blocking TNFR2 with utr-1, an antibody with antagonistic activity on the TNFR2 (Brockhaus et al., 1990; Shalaby et al., 1990), had no effect on TNF-induced ICAM-1 up-regulation. Interestingly, we found that at 4 h, TNF-inducible adhesion of T cells on ASM cells was exclusively mediated by the expression of ICAM-1 protein. Prior studies of T-lymphocyte adhesion to ASM found that adhesion to myocytes stimulated with cytokines for 24 h was more complex and involved multiple adhesion receptors, including ICAM-1, VCAM-1, and CD44 (Lazaar et al., 1994).

Signaling through the TNF receptor can proceed through multiple parallel pathways, leading to a wide range of biological activities. Activation of TNFR1 results in the recruitment of the TRADD (Hsu et al., 1995, 1996), whereas activation of TNFR2 leads to the recruitment of TRAF2 (Rothe et al., 1994). Recent data showed that TRADD also can interact with TRAF2 (Hsu et al., 1996) and, therefore, may explain why TRAF2 can mediate NF-κB activation by both TNFR1 and TNFR2. Our data suggest that in ASM cells, TNF-induced NF-κB activation is mediated by TNFR1 because htr-9, a specific receptor agonist, stimulated NF-κB-dependent reporter activity in transfected cells. Overexpression of the dominant negative form of TRAF2 abrogated htr-9- and TNFα-mediated NF-κB activation in ASM cells, which concurs with similar studies in other cell types. This provides indirect evidence for a requirement for TRAF2 in mediating TNF-induced ICAM-1 expression because NF-κB activation is necessary for this effect in smooth muscle (Amrani et al., 1999) and other cells (Staunton et al., 1988; Voraberger et al., 1991; Roebuck et al., 1995). We cannot rule out the possibility that other TNF receptor-associated signaling pathways such as lactosylceramide (Bhunia et al., 1998), phosphatidylinositol 3-kinase, or Akt (Béraud et al., 1999) mediate ICAM-1 expression. Finally, one also must be careful when interpreting these in vitro findings because TRAF2 −/− mice exhibit functional NF-κB activation after exposure to TNF (Lee et al., 1997; Yeh et al., 1997).

Thapsigargin, a specific and potent inhibitor of intracellular calcium pumps, i.e., sarco-endoplasmic reticulum calcium ATPase-type calcium ATPase, has been extensively used in a variety of cell lines to induce calcium signals by emptying intracellular stores without the generation of related second messengers (Thastrup et al., 1990). In ASM cells, activation of TNFR1 by TNFα "primes" airway myocytes to augment calcium transients evoked by contractile agonists as well as

to thapsigargin (Amrani et al., 1995b, 1996, 1997). Importantly, TNF alone did not evoke calcium transients over short time courses (Amrani et al., 1995b). Our previous work suggests that thapsigargin-sensitive calcium stores play an important role in mediating TNFα effects on calcium homeostasis (for review, see Amrani and Panettieri, 1998). In this study, we used thapsigargin to investigate whether stored calcium also modulates cytokine-induced gene expression in human ASM cells. We show that thapsigargin inhibited ~50% of TNFα-induced expression of ICAM-1 in a dose-dependent manner, which correlated with the magnitude of thapsigargin-induced calcium mobilization as shown in previous reports (Amrani et al., 1995a, 1996). These data suggest that cytokine-mediated ICAM-1 expression is affected by depleting calcium stores sensitive to thapsigargin. The role of intracellular calcium was supported by the observation that chelating the cytosolic calcium concentration with the intracellular calcium chelator BAPTA-AM (Tsien, 1980) abrogated ICAM-1 induction by TNFα. In endothelial cells, BAPTA-AM has been used to demonstrate the involvement of calcium in the induction of heme oxygenase-1 by TNFα (Terry et al., 1999). Together, these findings indicate that TNFα-mediated gene expression in ASM cells is intimately dependent on calcium stored in intracellular compartments that can be depleted by thapsigargin. This is an important finding because we have previously shown that thapsigargin-sensitive calcium pools are activated by contractile agonists to elicit cytosolic calcium signals and to regulate calcium influx in ASM cells (Amrani et al., 1995a, 1996). Thus, calcium accumulated within calcium-ATPase sarco-endoplasmic reticulum calcium ATPase-associated pools appears to regulate a variety of other functions in human ASM cells. The precise mechanisms by which thapsigargin modulates ICAM-1 expression remain unclear. In rat myocytes (Reilly et al., 1998) and NIH 3T3 fibroblasts (Aktas et al., 1998), emptying internal calcium stores inhibits protein synthesis by modulating protein translation initiation. In these studies, calcium mobilized from stores in response to clotrimazole (Aktas et al., 1998) or thapsigargin (Reilly et al., 1998) activated protein kinase R, which in turn phosphorylated and inactivated the translation initiation factor eIF2α. Similarly, in endothelial cells, mobilization of intracellular calcium by thapsigargin or the calcium ionophore A23187 suppressed the translation of type-1 plasminogen activator inhibitor mRNA induced by TNFα (Peiretti et al., 1997). In this study, we also found that thapsigargin suppressed cytokine-induced NF-κB-dependent gene transcription, an effect that was not due to modulation of cytokine-induced IκBα degradation or NF-κB nuclear translocation, crucial steps for NF-κB activation. Together, these data support the hypothesis that the filling state of intracellular calcium stores regulates ICAM-1 expression, possibly by interfering with NF-κB-mediated gene expression. This cross talk between the filling state of thaspigargin-sensitive stores and NF-κB signaling also has been studied in different cell lines. Thus, in HeLa cells, 293 or U937 cell lines, investigators showed that NF-κB can be activated by a variety of agents that increase cytosolic calcium concentration such as thapsigargin or sphingosine-1-phosphate and agents that induce an endoplasmic reticulum stress such as 2-deoxyglucose (Pahl and Baeuerle, 1996; Shatrov et al., 1997). The interrelationship between internal calcium stores and NF-κB signaling was further supported by the fact that overexpressing proteins that accumulate within the endoplasmic reticulum stimulates NF-κB activation (Pahl et al., 1996). The role of intracellular calcium in regulating NF-κB signaling by these various stimuli was demonstrated by using the calcium chelator BAPTA (this study), which completely prevented NF-κB activation. In contrast to data observed in these different cell types, we found that in human ASM cells, emptying internal calcium stores with thapsigargin does not activate NF-κB. This may explain why thapsigargin has very little effect on ICAM-1 expression that was previously shown to be NF-κB-dependent (Staunton et al., 1988; Amrani et al., 1999). In addition, we also found that TNFα-mediated NF-κB-dependent gene transcription was blocked by BAPTA or thapsigargin, an effect not observed in HeLa and U937 cell lines (Pahl and Baeuerle, 1996; Shatrov et al., 1997). This may be due to the fact that in human ASM cells and also in human endothelial cells (Peiretti et al., 1997), the intracellular stored calcium may serve as an important messenger in the signal transduction pathways for TNFα-induced NF-κB-dependent gene expression. This precise calcium-dependent pathway is currently being investigated. In addition, our previous study showed that nickel, an agent that blocks calcium influx, completely suppressed the activation of NF-κB induced by CD40 engagement, suggesting a role for extracellular calcium in NF-κB activation (Lazaar et al., 1998). In addition, our study demonstrates a close relationship between calcium originating from the sarco-endoplasmic reticulum and the regulation of gene expression by NF-κB in human ASM cells. This was not a generalized effect on protein transcription because β-actin mRNA expression was unaffected by thapsigargin pretreatment. Additional experiments are needed to determine the precise mechanisms by which calcium present in the internal stores modulates cytokine-induced ICAM-1 expression.

Together, our data show that in human ASM cells, TNFR1 is coupled to a TRAF2-NF-κB signaling pathway and plays a key role in regulating the rapid expression of functional ICAM-1. More importantly, we show that calcium originating from thapsigargin-sensitive stores regulates TNFR1-mediated ICAM-1 by promoting NF-κB-mediated gene transcription. Further experiments are needed to determine the molecular mechanisms by which cytosolic calcium regulates NF-κB action and gene expression in human ASM cells.

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