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NF-κB, But Not p38 MAP Kinase, Is Required for TNF-α-Induced Expression of Cell Adhesion Molecules in Endothelial Cells

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ABSTRACT

In response to inflammation stimuli, tumor necrosis factor-α (TNF-α) induces expression of cell adhesion molecules (CAMs) in endothelial cells (ECs). Studies have suggested that the nuclear factor-κB (NF-κB) and the p38 MAP kinase (p38) signaling pathways play central roles in this process, but conflicting results have been reported. The objective of this study is to determine the relative contributions of the two pathways to the effect of TNF-α. Our initial data indicated that blockade of p38 activity by chemical inhibitor SB203580 (SB) at 10 μM moderately inhibited TNF-α-induced expression of three types of CAMs; ICAM-1, VCAM-1 and E-selectin, indicating that p38 may be involved in the process. However, subsequent analysis revealed that neither 1 μM SB that could completely inhibit p38 nor specific knockdown of p38α and p38β with small interference RNA (siRNA) had an apparent effect, indicating that p38 activity is not essential for TNF-α-induced CAMs. The most definitive evidence to support this conclusion was from the experiments using cells differentiated from p38α knockout embryonic stem cells. We could show that deletion of p38α gene did not affect TNF-α-induced ICAM-1 and VCAM-1 expression when compared with wild-type cells. We further demonstrated that inhibition of NF-κB completely blocked TNF-α-induced expression of ICAM-1, VCAM-1 and E-selectin. Taken together, our results clearly demonstrate that NF-κB, but not p38, is critical for TNF-α-induced CAM expression. The inhibition of SB at 10 μM on TNF-α-induced ICAM-1, VCAM-1 and E-selectin is likely due to the nonspecific effect of SB. J. Cell. Biochem. 105: 477–486, 2008.

KEY WORDS: NF-κB; p38 MAP KINASE; TNF-α; CELL ADHESION MOLECULES; ENDOTHELIAL CELLS

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine produced by many types of cells and was originally identified on the basis of its ability to cause necrosis in certain cells. It is now known that TNF-α can elicit a wide range of pathological–physiological responses including inflammation, cell proliferation and differentiation, in addition to its ability to induce cell death [Beyaert and Fiers, 1994]. The pleiotropic effect of TNF-α is due to the fact that it activates multiple signaling pathways in different cells, which independently or coordinately regulates various cellular processes [Gaur and Aggarwal, 2003]. The wide variety of TNF-α elicited responses depends on cell types and their physiological states. Therefore, the signaling pathways that mediate the effects of TNF-α in different cells require specific investigation.

Endothelial cells (ECs) form the barrier between the blood and surrounding tissues. Under normal conditions, ECs maintain blood stream that allows the continuous flow of plasma and blood cells. During inflammation, ECs become activated and participate in inflammatory responses through the expression of pro-inflammation genes, including cytokines, chemokines and growth factors [May and Ghosh, 1998]. Among a wide variety of molecules induced, TNF-α elicits rapid expression of cell adhesion molecules (CAMs) at the surface of endothelium, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1). These molecules mediate transmigration of leukocytes from blood stream to the underlying inflammatory tissues. Depending on the anatomical locations and developmental stages of the vasculatures, the expression of CAMs in different types of ECs may vary [Aird, 2007]. For instance, E-selectin is expressed in most types of ECs in response to cytokines, but it is not inducible in embryonic endothelium [Milstone et al., 2000]. ICAM-1 can be induced in lung, kidney, liver, and heart, whereas VCAM-1 is also induced in these organs except in lung [Aird, 2007]. While these differences are well recognized among ECs of different origins, the vast majority of in vitro experiments use human umbilical vein ECs

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(HUVECs) and human microvascular ECs (HMVECs) since they are easy to obtain and to maintain in culture. As a result, our knowledge of the signaling pathways that regulate CAM expression at the cellular level is primarily derived from HUVECs, and to a lesser extent, HMVECs [Aird, 2007]. Even in these cells, the precise regulatory mechanisms are not well understood.

TNF-α activates several intracellular signaling pathways, of which the nuclear factor-κB (NF-κB) and the p38 MAP kinase (p38) pathways are the most studied [Gaur and Aggarwal, 2003]. NF-κB is a transcription factor that resides in the cytoplasm of unstimulated cells in an inactive form through its association with the inhibitory protein inhibitor-κB (I-κB). Cell stimulation by TNF-α triggers degradation of I-κB through proteolysis, which allows the translocation of NF-κB from cytoplasm to the nucleus where it activates gene transcription [May and Ghosh, 1998; Gaur and Aggarwal, 2003]. The members of p38 family, consisting of p38α, p38β, p38γ, and p38δ, regulate diverse cellular functions by phosphorylating transcription factors, such as AP-1, ATF-2 and CREB or other enzymes [Ono and Han, 2000]. p38α is widely expressed in most cell types and is the first isoform discovered for its role in endotoxin-induced inflammatory response and osmotic shock [Lee et al., 1994; Ono and Han, 2000]. In many cells, TNF-α simultaneously activates NF-κB and p38, which may independently or synergistically regulate the effect of TNF-α [Baud and Karin, 2001; Grivennikov et al., 2006], but the detailed mechanisms of their action and the relative contributions are not well understood. In the case of TNF-α-induced CAMs in ECs, a pivotal role for NF-κB has been convincingly demonstrated [Denk et al., 2001], but the involvement of the p38 pathway is controversial. For instance, it has been shown that TNF-α-induced ICAM-1 was inhibited by p38 inhibitors in HUVECs [Ju et al., 2003; Westra et al., 2005], but other reports suggested that similar treatments showed no effect [Pietersma et al., 1997; Goebeler et al., 1999; Zhou et al., 2007]. Likewise, studies by Fitau et al. [2006] and by Zhou et al. [2007] showed that p38 inhibitors did not affect TNF-α-induced VCAM-1, but other investigators reported otherwise [Pietersma et al., 1997; Ju et al., 2003; Lin et al., 2005]. Similar confusions have been reported for E-selectin [Read et al., 1997; Laferriere et al., 2001; Kuldo et al., 2005; Westra et al., 2005]. In epithelial cells, using an I-κBα negative mutant and SB inhibitors, Holden et al. [2004] convincingly demonstrated that TNF-α-induced ICAM-1 expression was highly NF-κB-dependent but p38-independent in A549 epithelial cell line. Whereas, in the same cells, Clarke et al. [2007] reported that TNF-α-induced ICAM-1 and VCAM-1 expression was significantly down-regulated by siRNA knockdown of p38 or by SB203580 inhibitors. Apparently, the same controversy regarding the role of p38 in TNF-α-induced CAMs reported in ECs also exists in epithelial cells.

Many of the aforementioned studies utilized pharmacological inhibitors of the p38 pathway. The most widely used in the literature are pyridinyl imidazole derivatives including SB203580 (SB), which effectively inhibits p38α and p38β, but not p38γ and p38δ [Cuenda et al., 1995; Gum et al., 1998]. However, nonspecific effects of SB to several other kinases have been reported [Davies et al., 2000; Lali et al., 2000; Coffey et al., 2002], which likely contributed to the above-described discrepancies in different studies. Therefore, an accurate assessment of the relative contributions of NF-κB and p38 to TNF-α-induced gene expression is not only important for understanding their cellular function, but also critical for the development of therapeutic drugs based on NF-κB and p38 inhibitors [Karin, 2005; Zhang et al., 2007]. Using small interference RNA (siRNA) in combination with inhibitors, we demonstrated that TNF-α-induced expression of CAMs did not require p38 activity. This conclusion was further confirmed in cells that were differentiated from p38α knockout embryonic stem cells in which p38α expression is completely eliminated at the gene level. Our data further revealed that NF-κB inhibitors blocked TNF-α-induced expression of VCAM-1, ICAM-1, and E-selectin under the conditions where p38 was actually activated. This interesting result is unexpected but further supports the conclusion that p38 is not required for TNF-α-induced VCAM-1, ICAM-1, and E-selectin expression whereas the activation of NF-κB is essential and sufficient for mediating the effect of TNF-α.

MATERIALS AND METHODS

ENDOTHELIAL CELL CULTURE AND CELL TREATMENT

Human umbilical vein ECs (HUVECs) and culture media were purchased from Clonetics. Cells were maintained in endothelial growth medium-2 (EGM-2) containing 5% fetal calf serum (FCS) at 37°C in a humidified incubator (5% CO_2, 95% air) as previously described [Yang et al., 2004]. Cells from three to eight passages were used. For experiments, EGM-2 was changed to M199 medium containing 1% FCS and cells were treated with human TNF-α 10 ng/ml (or otherwise indicated) under various conditions as specified in individual experiments.

MOUSE EMBRYONIC STEM CELL (ESC) CULTURE, DIFFERENTIATION, AND CHARACTERIZATION OF DIFFERENTIATED CELLS

Generation and characterization of mouse wild-type (p38α+/+) and p38α knockout (p38α−/−) ESCs have been previously described [Allen et al., 2000]. ESCs were maintained in complete ESC culture medium at 37°C in a humidified atmosphere at 5% CO_2, as described in detail in our previously published studies [Guo et al., 2007]. ESC differentiation was carried out according to published protocols [Yamashita et al., 2000; McCloskey et al., 2006] with some modifications. Briefly p38α+/+ and p38α−/− ESCs were seeded at the density of 10^4 cells/cm^2 in 25 cm flasks coated with 10 μg/ml Type IV collagen (BD Biosciences) and cultured in ESC medium for the first day, the medium was then switched to α-MEM-medium that contains 5% FCS and 5% serum supplement (Invitrogen). To increase the differentiation potential of ESCs to ECs, 20 ng/ml mouse VEGF and 20 ng/ml bFGF were added to the medium. After 5-day differentiation, the culture medium was changed to EGM-2 medium supplemented with 10 ng/ml VEGF and refreshed every other day. After 5-day incubation, differentiated cells formed confluent monolayers that contain enriched population of ECs. The cells were dissociated with trypsin and cultured in EGM-2 medium. Cells from two passages were used. For experiments to analyze TNF-α induced CAM expression, the cell treatments are essentially the same as described for HUVECs except that mouse TNF-α (Invitrogen) was used.

Immunocytochemical identification of ECs has been previously described in detail [Guo et al., 2007]. Briefly, fixed cells were permeabilized with PBS containing 0.25% Triton X-100 for 30 min. After being blocked in 2% bovine serum albumin and 5% preimmune serum, the cells were incubated with anti-PECAM-1 and anti-VE-cadherin antibodies (BD Biosciences). The positive cells were detected by fluorescein isothiocyanate (FITC) with a fluorescence microscope.

Matrigel in vitro tube formation assay was performed according to the method described by McCloskey et al. [2006]. Matrigel (300 μl, BD Biosciences) was added to wells of a 24-well plate and allowed to solidify for 30 min at 37°C. After matrigel was solidified, 5,000 ESC-differentiated cells were suspended in 0.5 ml EGM-2 medium and added to matrigel-coated wells. The cells were then incubated for 24 h and examined for tube (network) formation and photographed with a phase-contrast microscope.

siRNA TRANSFECTION OF HUVECs

siRNAs targeting p38α (5′-AAACAAUGUUCUUCCAGUCAACAGC-3′ and 5′-GCUGUUGACUGGAAGAACAUUGUUU-3′), p38β (5′-GCG-AAGUGUACUUGGUGAC-3′ and 5′-GUCACCAAGUACACUUCGC-3′), and negative control siRNA were obtained from Invitrogen. HUVECs at 60–70% confluence were transfected with siRNA at the final concentration of 20 nM using Lipofectamine (Invitrogen) or DharmaFECT (Dharmacon) according to the manufacturer's instructions. Five hours after transfection, the medium was replaced with fresh EGM-2. The cells were used for experiments after 30–48 h of further incubation.

RNA EXTRACTION AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RNA extraction and RT-PCR analysis have been described previously [Guo et al., 2007]. Briefly, total RNA was extracted from cells with Tri-reagent (Sigma). cDNA was prepared by reverse transcription using M-MLV reverse transcriptase (Sigma). The specificity of PCR products was determined by dissociation curve and confirmed by agarose gel electrophoresis. Quantitative real-time PCR (qRT-PCR) was performed using the SYBR green jumpstart TAQ readymix (Sigma) on a MX3000P Real-time PCR system (Stratagene). Sequences for the primer sets were as follows:

HUVECs:

β-actin, forward: 5′-CATGTACGTTGCTATCCAGGC-3′, reverse: 5′-CTCCTTAATGTCACGCACGAT-3′
ICAM-1, forward: 5′-AGAGGTCTCAGAAGGGACCG-3′, reverse: 5′-GGGCCATACAGGACACGAAG-3′
VCAM-1, forward: 5′-ATGCCTGGGAAGATGGTCG-3′, reverse: 5′-GACGGAGTCACCAATCTGAGC-3′
E-selectin, forward: 5′-GATGAGAGGTGCAGCAAGAA-3′, reverse: 5′CTCACACTTGAGTCCACTGAAG-3′

Mouse ESC-differentiated cells:

β-actin, forward: 5′-CATGTACGTAGCCATCCAGGC-3′, reverse: 5′-CTCTTTGATGTCACGCACGAT-3′
ICAM-1, forward: 5′-GGCATTGTTCTCTAATGTCTCCG-3′, reverse: 5′-GCTCCAGGTATATCCGAGCTTC-3′
VCAM-1, forward: 5′-CCAAATCCACGCTTGTGTTGA-3′, reverse: 5′-GGAATGAGTAGACCTCCACCT-3′

The mRNA level from qRT-PCR was calculated using the comparative Ct method [Pfaffl, 2001]. β-actin mRNA was used as a calibrator for the calculation of relative mRNA levels of tested genes as previously described [Guo et al., 2007].

CELL LYSATE PREPARATION AND WESTERN BLOT ANALYSIS

HUVECs were lysed in M-PER mammalian cell protein extraction buffer (Pierce) supplemented with a cocktail of protease inhibitors. After being kept on ice for 30 min, the extracts were centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was designated as the cell lysate and used for Western blot analysis as previously described [Guo et al., 2001].

RESULTS

ACTIVATION OF p38 AND NF-κB BY TNF-α

Upon TNF-α stimulation, p38 and NF-κB are quickly activated. The early activation of these two pathways in turn activates other enzymes or transcription factors, which ultimately regulate TNF-α-induced physiological responses [Gaur and Aggarwal, 2003]. Figure 1A shows the time course of TNF-α-induced p38 and NF-κB activation by Western blot analysis. Activation of p38 requires phosphorylation of a threonine and a tyrosine residue in its active site. Therefore, phosphorylation on these two residues, which can be detected by phospho-specific antibodies, has often been used to indicate its activation [Guo et al., 2001]. The phosphorylated p38 (pp38) was detected with an anti-pp38 antibody (Cell Signaling) at 5 min treatment and reached maximum at 15 min treatment; thereafter, their activities declined to the basal level. The activation of p38 was further assessed by the phosphorylation of heat shock protein 27 (HSP27, a downstream component of the p38 pathway) with anti-pHSP27 antibodies (Santa Cruz Biotechnology). The phosphorylated HSP27 (pHSP) was detected at the time points corresponding to p38 activation (pp38). NF-κB is retained in the cytoplasm of unstimulated cells by I-κB. TNF-α treatment induces I-κB degradation and results in NF-κB translocation from cytoplasm to the nucleus. Therefore, degradation of I-κB is often used as an indicator of early activation of the NF-κB pathway. As shown in Figure 1A, at 5 min treatment with TNF-α, I-κB was significantly reduced and was completely degraded by 15 min. It was re-synthesized 2 h after treatment. The above results represent typical activation patterns of p38 and NF-κB by TNF-α in ECs.

To evaluate the roles of p38 and NF-κB in mediating the effects of TNF-α, we used 10 μM SB and 100 μM PDTC (pyrrolidinedithiocarbamate, Sigma), the concentrations commonly used in the literature, to inhibit p38 and NF-κB, respectively. As shown in Figure 1B, p38 activation by TNF-α was indicated by the levels of pp38 and pHSP27. SB slightly reduced p38 phosphorylation (pp38) but completely inhibited p38 activity as judged by the level of pHSP27 (pHSP). Likewise, TNF-α-induced degradation of I-κB was

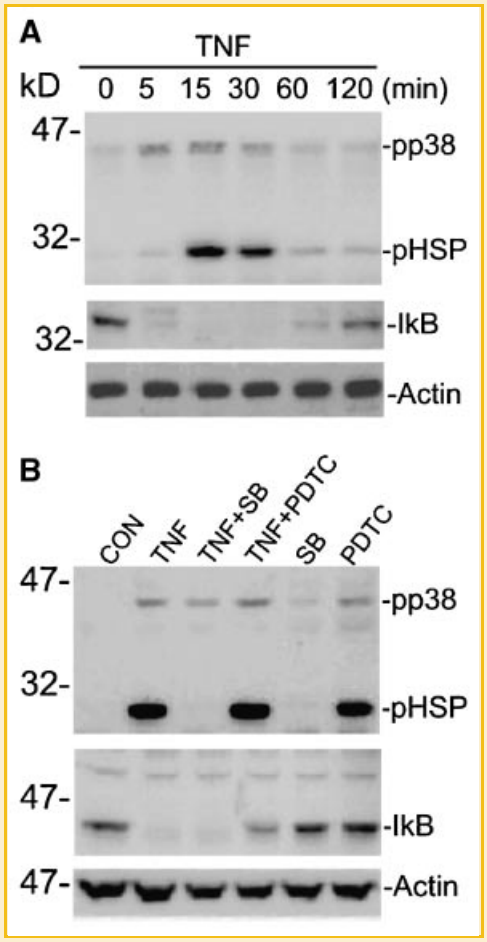

Fig. 1. Activation of p38 and NF-κB by TNF-α (A) and the effects of inhibitors (B). A: HUVECs were stimulated with TNF-α for the time indicated. p38 activation was detected by Western blot using anti-phospho-p38 antibodies that recognize both phospho-p38α/β (pp38) and by pHSP27. NF-κB activation was indicated by the degradation of I-κB. β-actin was used as a control for protein loading. B: Con, cells without TNF treatment; TNF, cells treated with TNF for 15 min; TNF + SB, cells pretreated with SB (10 μM) for 60 min followed by TNF for 15 min; TNF + PDTC, cells pretreated with PDTC (100 μM) for 60 min followed by TNF for 15 min; SB and PDTC, cells treated with SB or PDTC alone, respectively. p38 and NF-κB activation and protein loading were analyzed by the same methods as described in (A). The results are representative of similar experiments repeated three times. The molecular weight markers are indicated on the left side of the blots.

significantly attenuated by PDTC, which is widely used to inhibit the NF-κB pathway. As additional controls, we also tested the effect of SB and PDTC alone. It is interesting to note that PDTC by itself activated p38 as indicated by pp38 and pHSP27 while SB alone had no effect on either p38 activation or the I-κB degradation (Fig. 1B).

TNF-α-INDUCED CAM EXPRESSION AND THE EFFECTS OF p38 AND NF-κB INHIBITORS

The above experiments demonstrated the short-term responsiveness of HUVECs to TNF-α and the expected effects of SB and PDTC. Since an essential role of NF-κB in TNF-α-induced expression of CAMs has been well recognized, we focused on the investigation of the contribution of the p38 pathway to TNF-α-induced expression of three types of CAMs; ICAM-1, VCAM-1, and E-selectin. RNA isolated from treated cells for 24 h was analyzed with qRT-PCR. As shown in Figure 2, TNF-α strongly induced expression of all the three CAMs. Pretreatment of cells with 10 μM SB inhibited TNF-α-induced ICAM-1, VCAM-1, and E-selectin transcription by 40%, 34%, and 47%, respectively (Fig. 2, graph). We further confirmed the effect of SB on TNF-α-induced ICAM-1 expression by Western blot with an anti-ICAM-1 antibody (Santa Cruz Biotechnology) under the same experimental conditions. Consistent with qRT-PCR results, SB inhibited TNF-α-induced ICAM-1 at the protein level (Fig. 2, inset). In this experiment, we also tested PDTC, which significantly reduced TNF-α-induced ICAM-1 as expected. SB or PDTC alone had no effect on the expression of ICAM-1. Similar inhibitory effect of SB at 10 μM or above on TNF-α-induced ICAM-1 and/or VCAM-1 have been reported at the transcription or protein levels in ECs [Pietersma et al., 1997; Ju et al., 2003; Lin et al., 2005] and in epithelial cells [Woo et al., 2005; Clarke et al., 2007].

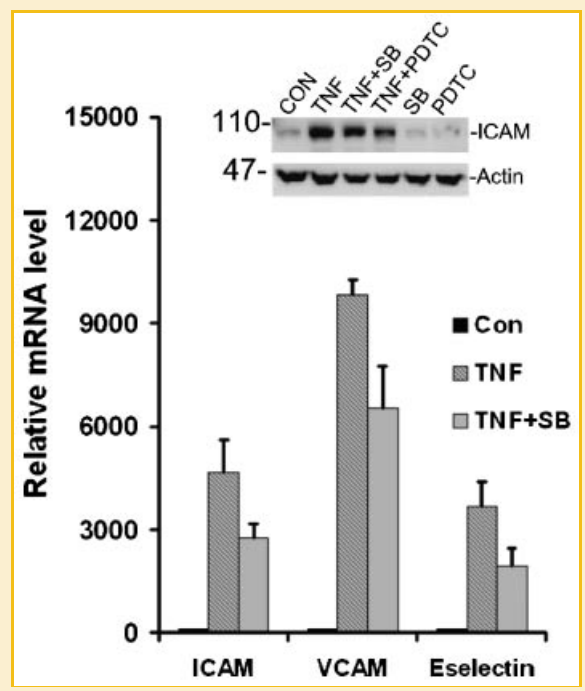

Fig. 2. TNF-α-induced CAM expression and effects of p38 and NF-κB inhibitors. qRT-PCR analysis of TNF-α-induced CAMs and effect of SB (graph): Con, cells without TNF-α treatment; TNF, cells treated with TNF-α for 24 h; TNF + SB, cells pretreated with SB (10 μM) for 60 min followed by TNF-α for 24 h. Total RNA was extracted from treated cells. The mRNA levels of CAMs were determined by qRT-PCR in triplicates. The mRNA of each gene determined from control experiment was set as 100%. Western blot analysis of ICAM-1 expression and effects of SB and PDTC (inset): Cells were treated under the same conditions as described for qRT-PCR analysis. TNF + PDTC, cells treated with PDTC (100 μM) for 60 min followed by TNF-α for 24 h; SB and PDTC, cells treated with SB or PDTC alone, respectively. The data are representative of two independent experiments with similar results.

EFFECTS of SB AT DIFFERENT CONCENTRATIONS ON TNF-α-INDUCED p38 ACTIVATION AND EXPRESSION OF CAMs

The results from the above experiments would indicate that p38 contributed to the expression of all three types of CAMs. Although reported with IC_{50} of 0.6 μM [Cuenda et al., 1995], SB is widely used at the concentrations of 5–20 μM in many in vitro experiments. To eliminate the nonspecific effect of SB on TNF-α-induced CAM expression, it is necessary to use SB at the lowest concentration that maximally inhibits p38 activity. Therefore, we examined the effect of SB on TNF-α-induced p38 activation at different concentrations. As shown in Figure 3A, SB inhibited TNF-α-induced p38 activation in a dose-dependent manner as judged by HSP27 phosphorylation (pHSP27). It exhibited a strong inhibitory effect even at the concentration as low as 0.1 μM with a complete inhibition at 1 μM. p38 phosphorylation (pp38) was only slightly reduced at all concentrations tested. This is explained by the fact that p38 is primarily phosphorylated by the upstream kinase MKK6, which is insensitive to SB. The slightly reduced pp38 is likely due to SB-inhibited p38 autophosphorylation [Kang et al., 2006]. We then tested the effect of SB at 0.5 and 1 μM SB on TNF-α-induced ICAM-1. As shown in Figure 3B, neither concentration of SB had an apparent effect on TNF-α-induced ICAM-1 at the protein level. The previous experiment analyzed the effect of 10 μM SB on TNF-α-induced mRNA of CAMs at a 24 h time point (Fig. 2). We performed the same experiment to test the effect of different concentrations of SB at 5 h after TNF-α treatment. As shown in Figure 3C, SB did not inhibit TNF-α-induced ICAM-1, VCAM-1 and E-selectin, either at 0.5 or 1 μM. It is interesting to note that, even at 10 μM, SB only inhibited VCAM-1, but not ICAM-1 or E-selectin expression at 5 h of TNF-α treatment (Fig. 3C). This is somewhat different from the results obtained from 24 h treatment at which the expression of all three CAMs was inhibited by 10 μM SB (Fig. 2B). These results indicate that SB (10 μM) could exert its effect at different steps of CAM expression in response to TNF-α and its effect may be observed at different time points depending on different CAMs. Since 1 μM SB is the minimal concentration that can completely inhibit p38 activation, examining the involvement of the p38 pathway in mediating the effect of TNF-α at this concentration may be able to reduce the nonspecific effect. At this concentration, SB did not affect CAM expression induced by 10 or 50 ng/ml TNF-α (Fig. 3D). We then performed a time course study, as shown in Figure 3E, SB did not have significant effect on TNF-α-induced CAM expression at time points tested except that VCAM-1 expression was moderately decreased at 9 and 12 h. Taken together, these results indicate that the p38 pathway is not critical for TNF-α-induced CAM expression although it might modulate the expression of VCAM-1 at certain steps.

KNOCKDOWN OF p38α AND p38β BY siRNA DID NOT AFFECT THE EXPRESSION OF TNF-α-INDUCED CAMs

The results from the above experiments indicate that the inhibitory effect of 10 μM SB on the expression of CAMs may be associated with nonspecific effects. To prove this hypothesis, we used specific p38α and p38β siRNA to knock down their expression. As shown in Figure 4A, treatment of HUVECs with p38α siRNA (Siα) and p38β siRNA (Siβ) effectively reduced the expression of each isoform as

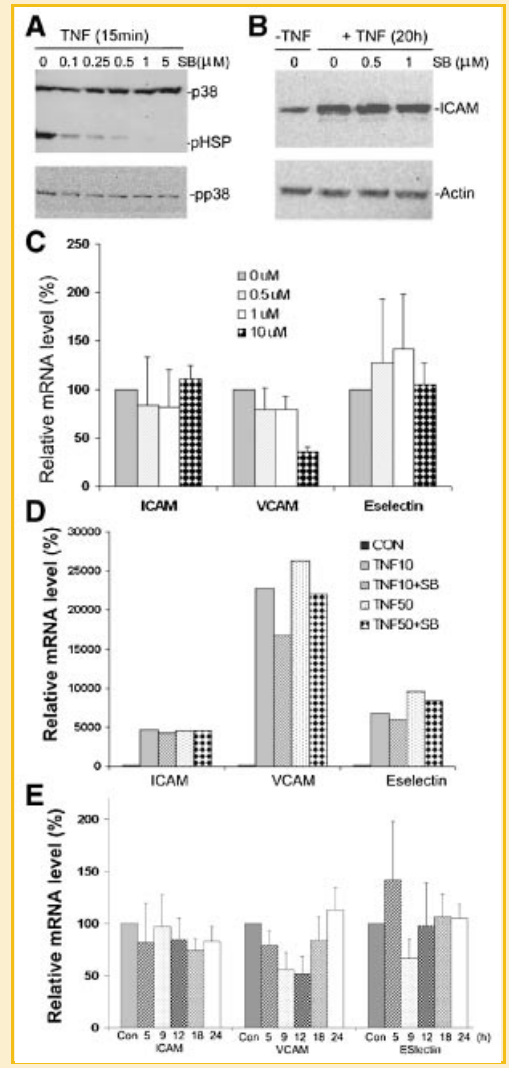

Fig. 3. Effects of SB at different concentrations on TNF-α-induced p38 activation and CAM expression. A: Inhibition of p38 activation by SB. Cells were treated with SB at different concentrations as indicated for 60 min followed by TNF-α for 15 min. p38 activation was determined by the levels of pHSP27 (pHSP) and pp38. The p38 protein as a loading control was detected with anti-p38 antibodies. B: SB at the concentrations that inhibits p38 does not affect TNF-α induced ICAM-1 expression. Cells were treated with SB (0.5 and 1 μM) for 60 min followed by TNF-α for 20 h. −TNF represents cells without TNF treatment. ICAM-1 was detected by Western blot with its antibodies. β-actin was used as a control for protein loading. C: Effect of different concentrations of SB on TNF-α-induced CAMs. Cells were treated with TNF-α for 5 h in absence (0 μM) or presence of SB (0.5, 1, or 10 μM). The mRNA levels of CAMs were determined by qRT-PCR. The mRNA level of each gene determined from control (SB 0) experiment was taken as 100%. Results are mean ± SD of three independent experiments. D: Effect of SB on different concentrations of TNF-α induced CAM expression. Cells were treated with 10 ng/ml or 50 ng/ml TNF-α in the absence (CON) or in the presence of 1 μM of SB for 5 h. The mRNA levels of CAMs were determined by qRT-PCR. The mRNA of each gene determined from control experiment was set as 100%. Results represent mean of duplicate assays. E: Effect of SB on TNF-α-induced expression of CAMs at different incubation times. Cells were treated with 10 ng/ml TNF-α in the absence (Con) or in the presence of 1 μM SB as indicated for different times. The mRNA levels of CAMs were determined by qRT-PCR. The mRNA of each gene determined from control experiments was set as 100%. Results are mean ± SD of three independent

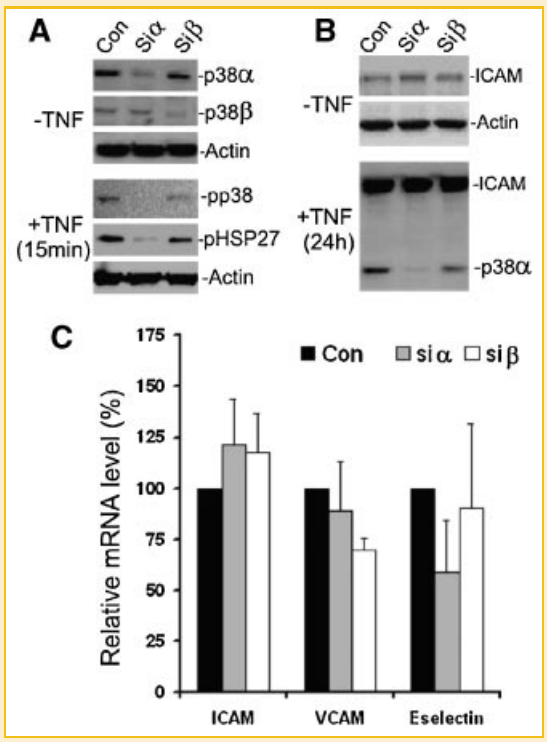

Fig. 4. siRNA down-regulation of p38α and p38β did not affect TNF-α-induced CAMs. A: Cells were treated with siRNA p38α (Siα), siRNA p38β (Siβ) or control siRNA (Con). The efficiency of p38 knockdown was analyzed at the protein level in the absence of TNF-α (−TNF) or at the activity level in the presence of TNF-α (+TNF) with the antibodies as indicated. Actin was used as a loading control. The experiment was repeated at least three times with similar results. B: Cells were treated with siRNAs as described in (A) followed by incubation with (+TNF) or without (−TNF) TNF-α for 24 h. The expression of ICAM-1 and p38α was determined by Western blot. The data are representative of the experiments performed three times with similar results. C: Cells were treated with siRNA as described in (A) followed by incubation with TNF-α for 5 h. The mRNA of each gene determined from control siRNA (Con) experiment was taken as 100%. Results are mean ± SD of three independent experiments.

determined with specific antibodies of p38α (Cell Signaling) and p38β (kindly provided by Dr. J. Han, the Scripps Institute) (Fig. 4A, −TNF). p38α siRNA treatment dramatically reduced pp38 and pHsp27 in response to TNF-α while p38β siRNA treatment showed a moderate effect (Fig. 4A). These results demonstrate the downstream effect of p38 knockdown. They also suggest that p38α is the dominant isoform that mediates the effect of TNF-α. We then examined TNF-α-induced ICAM-1 in cells treated with siRNA. Contrary to the inhibitory effect of SB observed at 10 μM (Fig. 2), knockdown of p38α or p38β affected neither TNF-α-induced ICAM-1 (Fig. 4B, +TNF) nor the basal level of ICAM-1 (Fig. 4B, −TNF). The effectiveness of knockdown of p38α by siRNA was confirmed in the same blot in which p38α was dramatically reduced (Fig. 4B). We further analyzed the effects of p38α and p38β knockdown on TNF-α-induced ICAM-1, VCAM-1 and E-selectin by qRT-PCR. Figure 4C shows that knockdown of p38α and p38β did not significantly affect mRNA levels of the three CAMs at 5 h treatment.

p38α KNOCKOUT DID NOT AFFECT TNF-α-INDUCED ICAM-1 AND VCAM-1 EXPRESSION IN ESC-DIFFERENTIATED CELLS

Although siRNA can specifically knock down their targets, a general concern is that they may be unable to completely repress the target gene expression and that the knockdown efficiency may vary from experiment to experiment. By using p38α knockout (p38α−/−) cells, in which p38α expression is completely eliminated at the gene level, we can avoid the shortfalls associated with siRNA approach. We have recently reported that, like p38α+/+ ESCs, p38α−/− ESCs can differentiate into ECs, smooth muscle cells and neurons [Guo et al., 2007]. In this study, we used a modified method that favors EC differentiation. Undifferentiated ESCs are small round cells that grow in colonies (Fig. 5A). After differentiation for 10 days, the cells derived from p38α+/+ and p38α−/− ESCs formed confluent monolayers without apparent differences (Fig. 5B, only p38α+/+ are shown). Although the differentiated cells contain other types of cells (primarily smooth muscle cells as judged by their expression of

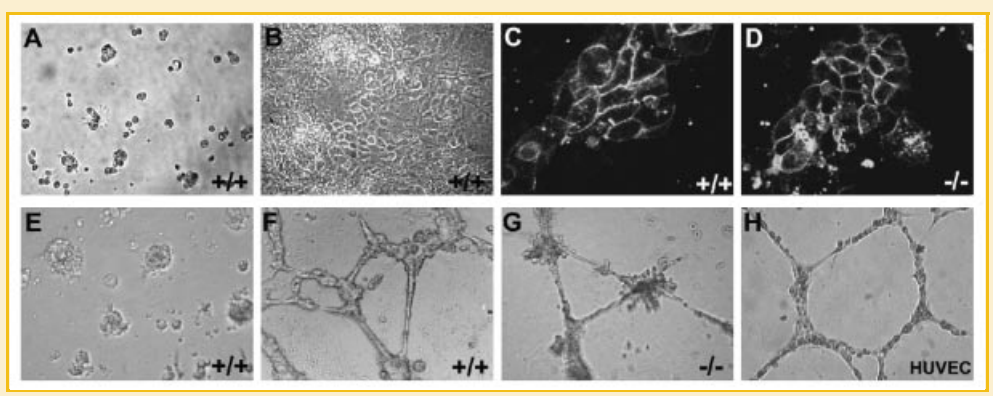

Fig. 5. Characterization of ESC-differentiated cells. A: Undifferentiated p38α+/+ESCs. B: The monolayers of differentiated p38α+/+ cells and p38α−/− cells have similar morphology (only p38α+/+ cells are shown). C,D: Identification of ECs by immunostaining of PECAM-1 in differentiated p38α+/+ and p38α−/− cells. E: Undifferentiated p38α+/+ and p38α−/− ESCs grow in aggregates on matrigel (only p38α+/+ ESCs are shown). F–H: Differentiated p38α+/+ and p38α−/− cells form network structures on matrigel after 24 h incubation, similar to HUVECs that were used as a positive control. The cells were examined and photographed with either a CKx31SF Olympus phase contrast microscope (100×, A,B,E–H) or with a LSM 510 confocal microscope (400×, C,D).

smooth muscle α-actin, data not shown), a large population of cells is ECs that can be identified by their expression of EC specific markers PECAM-1 (Fig. 5C,D) and VE-cadherin (data not shown). Forming tube (network)-like structures on matrigel is an important feature of ECs [Vailhe et al., 2001] and has been used to characterize the ESC-derived ECs [McCloskey et al., 2006]. Undifferentiated ESCs formed large clumps on matrigel (Fig. 5E) while differentiated p38α+/+ and p38α−/− cells (Fig. 5F,G) assembled into network structures (Fig. 5E–G), similar to HUVECs (Fig. 5H) that was used as a positive control. These results are in agreement with our previous conclusion that deletion of p38α does not compromise the ability of ESCs differentiation to ECs [Guo et al., 2007]. More importantly, undifferentiated ESCs do not respond to TNF-α [Allen et al., 2000] whereas our differentiated cells acquired this ability.

In response to TNF-α, ESC-derived cells expressed ICAM-1 and VCAM-1 but not E-selectin, which may be due to incomplete maturation. These cells were also less responsive to TNF-α treatment in short-term (5 h). Therefore, we analyzed TNF-α-induced ICAM-1 and VCAM-1 expression in cells treated for 24 h. As shown in Figure 6A, p38α deletion does not affect the effect of TNF-α since similar levels of ICAM-1 and VCAM-1 were induced in p38α+/+ and p38α−/− cells. To test whether p38β plays a role in mediating the effect of TNF-α, we treated the cells with 1 μM SB, which inhibits both p38α and p38β. SB did not significantly alter the expression of ICAM-1 and VCAM-1 either in p38α+/+ (Fig. 6B) or in p38α−/− cells (data not shown). These results confirm that neither p38α (judged from knockout cells) nor p38β (judged from SB treatment) is required for TNFα-induced ICAM-1 and VCAM-1 expression.

NF-κB INHIBITORS BLOCKED TNF-α-INDUCED EXPRESSION OF CAMs WITH CONCURRENT p38 ACTIVATION

It is interesting to note that PDTC by itself strongly activated p38 (Fig. 1B). This result prompted us to further investigate how the effect of PDTC on p38 correlates with its inhibition of TNF-α-induced CAMs. We first analyzed the long-term effect of PDTC alone or in combination with TNF-α on p38 activation. As shown in Figure 7A, PDTC alone strongly activated p38 at 24 h incubation (PDTC). p38 activation in cells treated with TNF-α alone was very low and was virtually the same as in the control experiment. However, strong p38 activation was detected in cells treated with the combination of PDTC and TNF-α (TNF + PDTC). It is noted that this was the same condition under which the expression of ICAM-1 was inhibited by PDTC (Fig. 2, blot). A time course analysis revealed that PDTC activated p38 as early as 30 min exposure as judged by the phosphorylation of HSP27 and it lasted to the entire course of the experiment (2 h, Fig. 7B). p38 activation was detectable at 50 μM PDTC treatment and reached maximal at 100 μM (Fig. 7C). To test if there is a correlation between PDTC induced p38 activation and NF-κB inhibition, we analyzed different concentrations of PDTC on TNF-α stimulated CAM expression. PDTC inhibited TNF-α-induced expression of E-selectin, ICAM-1 and VCAM-1 in a dose-dependent manner, which correlates but is not proportional to p38 activation level (data not shown).

We further tested another commonly used NF-κB inhibitor, MG-132 (MG, benzyloxycarbonyl-leucinyl-leucinyl-leucinal), a peptide-based proteasome inhibitor that blocks NF-κB activation by preventing I-κB degradation [Lee and Goldberg, 1998]. As shown in Figure 7D, MG inhibited TNF-α-induced I-κB degradation and caused accumulation of phospho-I-κB [Lee and Goldberg, 1998] (upper band, TNF + MG) as expected. It also activated p38 with a similar time course to PDTC. The inhibitory effect of PDTC and MG on TNF-α induced CAMs was further demonstrated at the mRNA level. Figure 7E illustrated that 10 μM MG or 100 μM PDTC completely inhibited the TNF-α-induced expression of E-selectin, ICAM-1 and VCAM-1. This result, similar to that obtained with genetic approaches [Denk et al., 2001; Jiang et al., 2004; Viemann et al., 2004; Kuldo et al., 2005], suggested that the NF-κB pathway is essential and sufficient for TNF-α-induced CAM expression whereas p38 activity is not critical.

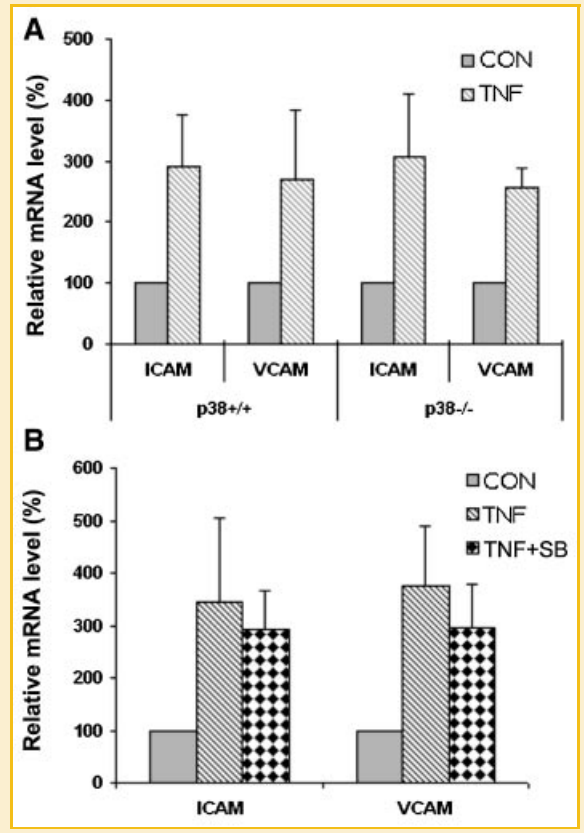

Fig. 6. TNF-α-induced CAM expression in ESC-differentiated p38α+/+ and p38α−/− cells and effects of SB. A: ESC-differentiated p38α+/+ and p38α−/− cells were treated with (TNF) or without (CON) 20 ng/ml mouse TNF-α for 24 h. B: ESC-differentiated p38α+/+ cells were treated with (TNF) or without (CON) 20 ng/ml mouse TNF-α for 24 h. To test the effect of SB, cells were treated with SB (1 μM) for 60 min followed by TNF-α (TNF + SB) for 24 h. The mRNA of each gene determined from control siRNA (CON) experiments was taken as 100%. Results are mean ± SD of four independent experiments.

DISCUSSION

The involvement of NF-κB and p38 in the regulation of inflammatory responses has been intensively investigated. However,

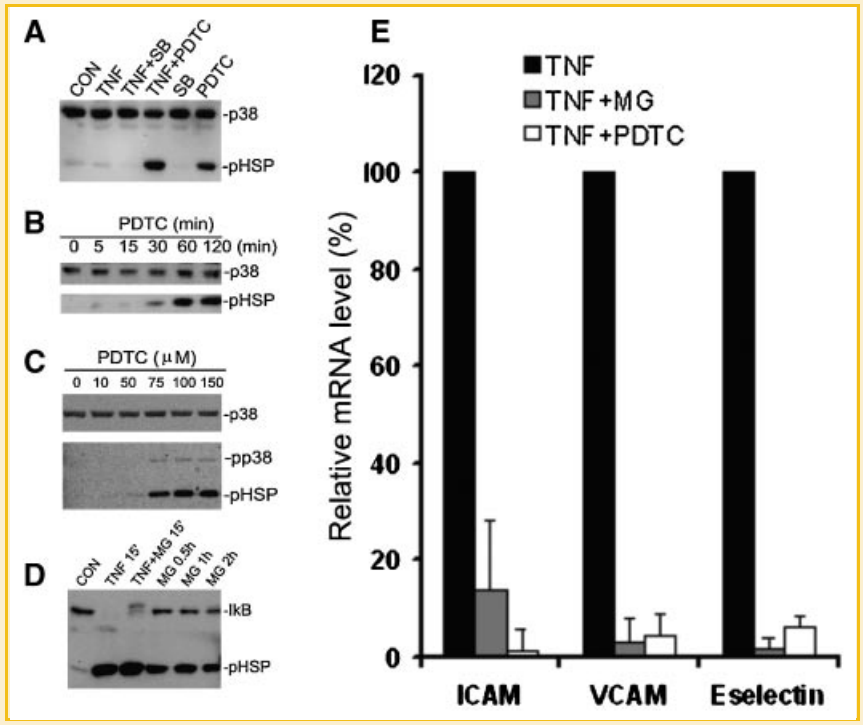

Fig. 7. NF-κB inhibitors activated p38 and blocked TNF-α-induced CAM expression. A: Activation of p38 by PDTC in long-term treatment. Cells were treated with TNF-α, SB, PDTC, or in combinations as indicated for 24 h under the same condition described in Figure 2. p38 activation was indicated by pHSP27 (pHSP). The p38 protein was detected with anti-p38 antibodies as loading control. B: Time course of p38 activation by PDTC (100 μM). C: Dose-dependent activation of p38 by different concentrations of PDTC in cells treated for 2 h. D: MG inhibited I-κB degradation and resembled PDTC in p38 activation. CON, control cells without treatment; TNF, cells treated with TNF-α for 15 min; TNF + MG, cells pretreated with MG (10 μM) for 60 min followed by TNF-α for 15 min; MG, cells treated with MG for 0.5, 1, or 2 h. NF-κB inhibition was indicated by I-κB degradation. MG inhibited TNF-α-induced I-κB degradation and caused accumulation of phospho-I-κB (upper band, TNF + MG). p38 activation was indicated by pHSP27 (pHSP). E: MG and PDTC completely inhibited TNF-α induced CAMs. TNF, cells treated with TNF-α for 5 h as control; TNF + MG, cells treated MG (10 μM) for 60 min followed by TNF-α for 5 h; TNF + PDTC, cells treated with PDTC (100 μM) for 60 min followed by TNF-α for 5 h. The mRNA levels of CAMs were analyzed by qRT-PCR. The mRNA level of each gene determined from control experiments (TNF) was taken as 100%. Results are mean ± SD of three independent experiments.

understanding their precise roles is a challenging task since inflammation involves numerous cytokines, growth factors, and chemokines, many of which often simultaneously activate p38 and NF-κB in many cells. Furthermore, the degree of activation of the two pathways may vary depending on cell types and the nature of stimuli. As a result of these complications and the different experimental approaches used, inconsistent and even conflicting results have been reported. This study intends to clarify the confusion regarding the relative contributions of the NF-κB and p38 pathways to TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin by a combination of different approaches.

Our initial results showed that 10 μM SB inhibited TNF-α induced expression of ICAM-1, VCAM-1, and E-selectin in HUVECs. Similar observations have been reported by other investigators in ECs [Pietersma et al., 1997; Ju et al., 2003; Lin et al., 2005] or in epithelial cells [Woo et al., 2005; Clarke et al., 2007]. These results would indicate that p38 was required in mediating TNF-α-induced CAMs. Although identified as a specific inhibitor for p38α and p38β with IC_{50} of 0.6 μM [Cuenda et al., 1995], SB is widely used at the concentrations of 5–20 μM. Like many other chemical inhibitors, nonspecific effect of SB on other kinases has been reported [Davies et al., 2000; Lali et al., 2000; Coffey et al., 2002]. It is possible that the inhibitory effect of SB at 10 μM on TNF-α-induced CAM expression in HUVECs could be associated with the off target effect; thereby, contributing to the discrepancies reported in different studies. This speculation was first indicated by the results that SB at 1 μM completely blocked p38 activation without affecting TNF-α-induced ICAM-1 and E-selectin expression although it slightly reduced VCAM-1 expression at 9 and 12 h treatment. This result indicates that overall p38 plays an insignificant role in mediating the effect of TNF-α. The results from siRNA experiments further support this conclusion. We recognized the fact that p38 expression may not be completely knocked down by siRNA, thus, its contribution to the effect of TNF-α might be underestimated. However, our data derived from p38α−/− cells, in which p38α expression is completely eliminated at the gene level, clearly demonstrated that p38 activity is not essential for TNF-α-induced ICAM-1 and VCAM-1 expression since similar results were obtained from wild-type and p38α−/− cells, and that SB at 1 μM did not affect the effect of TNF-α as found in HUVECs. The present study used a modified ESC differentiation method that significantly enriched EC differentiation. Differentiated ECs display the important features of mature ECs and are responsive to TNF-α. However, we recognized the fact that the ESC-differentiated cells are not a pure EC population and that E-selectin was not induced in response to TNF-α as described by other investigators [Milstone et al., 2000].

Nevertheless, we believe that these factors do not prevent us from drawing the above stated conclusion.

The essential role of the NF-κB pathway in TNF-α-induced expression of CAMs has been convincingly demonstrated by studies using pharmacological inhibitors and by genetic approaches [Denk et al., 2001; Jiang et al., 2004; Viemann et al., 2004; Kuldo et al., 2005]. A study by Denk et al. [2001] showed that over-expressing dominant negative I-κB, which is resistant to proteolysis, completely blocked TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin. They concluded that the NF-κB pathway was both essential and sufficient for the TNF-α effect, which would imply that other signaling pathways, including the p38 pathway, was not required. A recent report by Viemann et al. [2004] further supports this conclusion. Using microarray method, they identified 58 out of 13,000 genes that were significantly up-regulated by TNF-α in HUVECs. Virtually all of these genes depended on NF-κB activity as demonstrated by the expression of a dominant-negative mutant of IKK2. Only the expression of 13 genes appeared to be modulated by p38. It seems that the role of p38 pathway in mediating the effect of TNF-α in ECs is not as critical as it was originally thought [Viemann et al., 2004]. Our results are in agreement with these studies regarding the essential role of NF-κB in mediating TNF-α-induced expression of CAMs. They also clearly indicate that the p38 pathway is not essential for TNF-α-induced CAM expression.

Recent studies have shown that both PDTC and MG-132 themselves can activate p38 and AP-1 transcription activity [Hartsfield et al., 1998; Wu et al., 2004], but the implication of this finding in the context of these compounds as NF-κB inhibitors and their effects on TNF-α regulated gene expression was not evaluated. Therefore, we investigated how TNF-α-activated p38 activity was affected when NF-κB was blocked by these inhibitors. We showed that both PDTC and MG strongly inhibited TNF-α-induced NF-κB activation and subsequent expression of ICAM-1, VCAM-1, and E-selectin, concurrent with strong p38 activation. An interesting question is whether the p38 activation caused by PDTC or MG is associated with NF-κB inhibition. Mechtcheriakova et al. [2001] showed that over-expressing dominant negative IKK2, the kinase that phosphorylates I-κB, blocked TNF-α-induced NF-κB activation but had no effect on p38 activity in HUVECs. This result suggests that our observation of PDTC- or MG-induced p38 activation is a separate event from their inhibition of TNF-α-activated NF-κB pathway. Once NF-κB is inhibited, TNF-α-induced CAM is blocked regardless of the activation status of the p38 pathway. Apparently, PDTC and MG not only inhibit NF-κB, but also activate p38 through a mechanism (s) that we currently do not know. These agents have been useful tools to identify the role of the NF-κB pathway in mediating the effect of TNF-α, but their nonspecific effects must be carefully considered like any other chemical inhibitors. Nevertheless, unlike in the case of SB, the critical role of NF-κB in TNF-α-induced gene expression initially identified by PDTC has been convincingly confirmed by genetic approaches [Denk et al., 2001; Jiang et al., 2004; Viemann et al., 2004; Kuldo et al., 2005].

Several drugs for treatment of inflammatory disorders based on NF-κB and p38 inhibitors have been developed or are undergoing clinical trials [Lee et al., 2000; Karin, 2005; Zhang et al., 2007]. It is now becoming increasingly important to understand the relative contributions of the NF-κB and the p38 pathways to TNF-α-induced responses. Our data further emphasize that the conclusions based on the results from pharmacological inhibitors have to be sustained by the results from other approaches. A careful consideration of their off-target effects is critically important to develop specific drugs for the treatment of inflammatory diseases.

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