Tumor necrosis factor activates CRE-binding protein through a p38 MAPK/MSK1 signaling pathway in endothelial cells

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Tumor necrosis factor α (TNF-α) is a potent multifunctional hormone produced predominantly by activated macrophages (6, 39, 62). TNF induces the hemorrhagic necrosis and regression of tumors in animals, is cytotoxic to some transformed and normal cell types, and promotes immunity, inflammation, insulin resistance, hypotension, shock, and, in some chronic diseases, the syndrome of wasting and malnutrition known as cachexia.

The endothelium is an important target for TNF action (25, 53). TNF increases the expression of major histocompatibility class I antigens and stimulates the production of chemotactic factors and other cytokines by the endothelium. TNF also induces the expression of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin, on the surface of endothelial cells. The acquisition of surface adhesive properties by neutrophils and lymphocytes is important for inflammatory processes dependent on transendothelial cell migration. TNF alters the barrier function and permeability of endothelial cell monolayers to macromolecules and may promote or inhibit angiogenesis, at least in part by inducing expression of tissue factor (10, 11, 20, 40, 49). TNF selectively targets tumor endothelium by acting in synergy with tumor-derived growth factors, such as vascular endothelial growth factor (VEGF), to induce high-level expression of tissue factor (10), in vivo coagulation, and tumor necrosis. TNF also targets tumor vasculature by diminishing activation of integrin αvβ3, an adhesion receptor found on proliferating endothelial cells, which plays a role in tumor angiogenesis (57). The capacity of TNF to target tumor vasculature has been used in cancer therapy, and administration of TNF to patients with locoregional disease causes rapid and complete disruption of the vasculature of melanoma metastases (42). The effects of TNF on normal and tumor endothelium make it important to understand how TNF mediates its effects in endothelial cells.

TNF initiates its actions by binding to one of two receptors, the 55-kDa type 1 TNF receptor (TNFR-1) or the 75-kDa type 2 TNF receptor (TNFR-2) (2, 8, 32, 43, 44, 59, 63). The extracellular domains of the receptors share homologies with one another and a group of cell surface receptors that include the FAS antigen, the low-affinity nerve growth factor receptor, the FAS antigen, the low-affinity nerve growth factor receptor, and TNF receptor-associated factors (TRAFs) (39, 71). These proteins play an oblique role in permitting TNFR-1 to activate caspase cascades that promote cell death and nuclear factor κB (NF-κB), a transcription factor that induces the expression of genes important to immunity and cell survival. Less is known of TNFR-2 signal transduction, although it binds TRAFs and has the capacity to activate NF-κB.

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The observations just enumerated show that TNF activates signaling events that affect cell viability and the activation of new gene transcription (39, 71). These events are probably interrelated, as many TNF responsive gene products have antiapoptotic functions. Two groups of transcription factors with central roles in inflammation, NF-xB and AP-1, are direct targets for TNF-induced signaling events (39, 71). Several other transcription factors including IRF1, NF-IL6, and EGR1 are affected by TNF (71). The multiplicity and complexity of the cellular effects of TNF make it likely that this list is not complete.

The cyclic AMP response element (CRE)-binding protein CREB activates the transcription of target genes in response to diverse stimuli, including peptide hormones, growth factors, and neuronal activity (60). CREB is important for the induction of cell proliferation, differentiation, adaptive responses, and hormonal control of metabolic processes. Phosphorylation of CREB on serine 133 increases its transcriptional activity. This phosphorylation does not alter the binding of CREB to CRE but rather increases its association with adaptor proteins, such as CREB-binding protein, and thereby plays an obligate role in activation of the transcriptional machinery (60). Phosphorylation and activation of CREB can be affected by activation of ERK, protein kinase C, calcium/calmodulin-dependent protein kinases, and p38 mitogen-activated protein kinases (22, 61, 67, 72). Considering the important role of TNF in metabolic changes associated with invasive stimuli and alterations of cell growth (70) and its significant effects on endothelial cells, we decided to test whether TNF affects the activity of CREB in human umbilical vein endothelial cells (HUVEC) and, if so, to characterize the mechanism through which such activation is affected. The possibility that TNF might affect CREB was suggested to us by a previous report showing that TNF induces CREB DNA binding (36). However, phosphorylation of CREB on serine 133 increases its transcriptional activity without altering the binding of CREB to CRE. Rather, phosphorylation on serine 133 increases the association of CREB with adapter proteins such as CREB-binding protein, and this leads to activation of the transcriptional machinery (60). The present study shows that TNF not only induces CREB DNA binding as was previously reported (36) but also induces CREB phosphorylation and transcriptional activation through a p38 MAPK/MSK1 signal transduction pathway in HUVEC.

**Materials and Methods**

**Materials.** Human recombinant TNF-α was a gift from Genentech (South San Francisco, CA). Dominant negative A-CREB and CREBm1 were gifts from David D. Ginty (Johns Hopkins University School of Medicine, Baltimore, MD). Antibodies to phosphorylated CREB/ATF-1 and CREB were from Cell Signaling Technology (South San Francisco, CA). The p38 MAPK kinase 2 and recombinant ATF-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). The CRE and mutant CRE double-stranded DNA oligonucleotides were purchased from Santa Cruz Biotechnology. The CRE oligonucleotide was end-labeled with [γ-32P]UTP using the T4 polynucleotide kinase and purified by passage through G25 spin column. To initiate formation of DNA protein complexes, 6 μg of each nuclear extract, binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM DTT, and 5% glycerol), and 1 μg of recombinant ATF-1. Immunoprecipitated proteins were fractionated on 8% polyacrylamide gels, and Western blots were probed with antibodies to p-CREB/ATF-1.

**Electrophoretic mobility shift and gene reporter assays.** Nuclear protein extracts were prepared using the NE-PER kit by cell lysis and centrifugation according to the directions of the manufacturer (Pierce). The CRE and mutant CRE double-stranded DNA oligonucleotides were purchased from Santa Cruz Biotechnology. The CRE oligonucleotide was end-labeled with [γ-32P]UTP using the T4 polynucleotide kinase and purified by passage through G25 spin column. To initiate formation of DNA protein complexes, 6 μg of each nuclear extract, binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM DTT, and 5% glycerol), and 1 μg of recombinant ATF-1. Immunoprecipitated proteins were fractionated on 8% polyacrylamide gels, and Western blots were probed with antibodies to p-CREB/ATF-1.

**RESULTS**

TNF regulates cellular gene expression, thereby permitting cells to adapt to their environment, and has significant effects on the endothelium. TNF modulates the DNA-binding activity of CRE-binding proteins (36), a group of related transcription factors that includes CREB, in human endothelial cells. However, the signaling pathway through which TNF induces CREB DNA binding and whether this leads to increased transactivation of CREB in endothelial cells has not been determined. The experiments reported here were aimed at addressing these issues. In pursuit of these goals, HUVEC were stimulated with...
TNF, and a Western blot prepared from lysed cells was probed with an antibody that recognizes CREB phosphorylated on serine 133. ATF-1 phosphorylated on serine 63, which migrates as a lower molecular weight species than phosphorylated CREB, is recognized by this antibody because the consensus phosphorylation sequences in CREB and ATF-1 are completely homologous (33, 47, 55). Here we found that TNF induced the time-dependent phosphorylation of serine 133-phosphorylated CREB and ATF-1-phosphorylated on serine 63 (Fig. 1A).

Phosphorylation of serine 133 in CREB does not alter the DNA binding of CREB to CRE but increases its association with adapter proteins, such as the CREB-binding protein, leading to activation of transcription (60). Therefore, the demonstration that treatment of HUVEC with TNF leads to phosphorylation of CREB led us to determine whether CREB transcriptional activity was increased by TNF. A gene reporter assay in which HUVEC were transfected with the 3XCRE luciferase reporter permitted the observation that TNF induces a 2.5-fold increase over the basal level of CREB transcriptional activity (Fig. 1B). To demonstrate that CREB was the transcription factor that was mediating the TNF effect, experiments were conducted with two dominant negative forms of CREB (Fig. 1B). One of the constructs, A-CREB, is a potent and specific inhibitor of CREB DNA binding (3). The other construct, CREBm1, binds to CREB-binding sites in DNA but is not activated because the transcriptional regulatory residue, serine 133, is mutated to alanine (24). Expression of either dominant negative form of CREB impaired the ability of TNF to transactivate CREB.

To determine whether one, the other, or both TNF receptors were responsible for initiating the signal that led to CREB phosphorylation, HUVEC were treated with antibodies that antagonize signaling through either TNFR-1 or TNFR-2. As shown in Fig. 2A, neutralization of signal transduction through TNFR-1 substantially inhibited the ability of TNF to promote CREB phosphorylation. Neutralization of signaling through TNFR-2 somewhat inhibited CREB phosphorylation, and the blockade of both receptors entirely inhibited TNF-induced activation of CREB. These observations suggest that TNFR-1 plays the most significant role in activation of CREB and that TNFR-2 may play a lesser role in induction of CREB phosphorylation by TNF.

The experiment just described was independently repeated three times, and the results were normalized to account for small variances in protein loading, averaged, and plotted as fold-activation relative to control. The results in Fig. 2B confirm that the neutralizing antibody directed against TNFR-1 significantly inhibits CREB phosphorylation. The data in Fig. 2B, considered in conjunction with the observations in Fig. 2A, indicate that if TNFR-2 is involved in activation of CREB by TNF, it plays a lesser role than TNFR-1 and its function would be difficult to validate in a statistically significant manner. Thus TNFR-1 is the dominant receptor used by TNF to induce signaling events that lead to CREB activation.

A control experiment was conducted to confirm that anti-TNFR-1 and anti-TNFR-2 were effective in blocking TNF signaling through each receptor. Cells were treated with either neutralizing antibody and then with medium or TNF before NF-kB DNA binding was assayed by EMSA. An antibody...
lysates were then probed with anti-phospho MAPK (Fig. 3A) and p38 MAPK (Fig. 3B). TNF promoted the time-dependent activation of MAPK and p38 MAPK in HUVEC. This observation led us to test the ability of pharmacological inhibitors of these signaling pathways to abrogate TNF-induced CREB phosphorylation. As illustrated in Fig. 4A, SB-203580, an inhibitor of p38 MAPK signaling, completely blocked TNF-promoted phosphorylation of CREB. In contrast with this observation, results shown in Fig. 4B demonstrate that inhibition of the MAPK signaling pathway using PD-98059 had no effect on the induction of CREB phosphorylation by TNF. Similarly, wortmannin, an inhibitor of PI 3-kinase, was without effect on TNF-promoted CREB phosphorylation. To still more rigorously exclude the possibility that MAPK, as opposed to p38 MAPK, plays a role in CREB activation by TNF, a second inhibitor of this signaling pathway, U-0126, was evaluated (16). As shown in Fig. 4C, this highly specific inhibitor of MAPK signaling was unable to inhibit TNF-induced activation of CREB in HUVEC.

CREB binds to a specific sequence, 5'-TGCGTCA-3', known as CRE. An EMSA showed that stimulation of HUVEC with TNF promoted formation of two predominant protein-DNA complexes (Fig. 5A). Supershifting with anti-CREB showed that CREB was a component of one of the DNA-protein complexes induced by TNF. As ATF2/cJun binds the same CRE as CREB (35), we determined whether any of the retarded DNA binding complexes contained these species. The slower migrating complex contained ATF2/cJun, based on supershifting with ATF2 and c-Jun antibodies. We also consistently observed a rapidly migrating band of unknown composition after treatment of HUVEC with TNF. Most significantly, TNF-induced CREB DNA binding (Fig. 5A) and gene reporter activity (Fig. 5B) were blocked by pretreatment of HUVEC with SB-203580.

The observations described above indicate that p38 MAPK signaling mediates the capacity of the TNF to promote CREB phosphorylation, DNA binding, and transactivation. Support for this conclusion comes from experiments in which confocal microscopy was used to examine TNF-induced phosphorylation of CREB (Fig. 6). HUVEC were treated with vehicle or SB-203580 and then stimulated with TNF. Confocal microscopy shows that phosphorylation of nuclear CREB/ATF1 was induced by TNF and abrogated by inhibition of the p38 MAPK signaling pathway.

MAPKAP kinase 2, MAPKAP kinase 3, and MSK-1 are CREB and ATF-1 kinases activated by p38 MAPK (14, 54, 67, 74). As antibodies to the phosphorylated forms of each of these kinases are not available, their activation was assayed by use of an in vitro kinase assay in which ATF-1 was the substrate (47). In this assay, HUVEC were treated with vehicle or TNF for 15 min and then MAPKAP kinase 2, MAPKAP kinase 3, or MSK-1 were immunoprecipitated from cell lysates and incubated with recombinant ATF-1. A Western blot was then probed with anti-phospho-CREB/ATF-1, and TNF was found to have induced significant activation of only MSK1 (Fig. 7A). In addition, activation of MSK1 by TNF was inhibited by SB-203580, indicating that a p38 MAPK/MSK-1 signaling pathway promotes CREB phosphorylation induced by TNF in
Attempts to demonstrate immunoprecipitation of equal amounts of MSK1, MAPKAPK2, and MAPKAPK3 in the experiment just described by Western blotting were unsuccessful even when using antibodies to the kinases from different suppliers. This failure was probably due to low expression of the kinases in HUVEC. However, we were able to confirm input of equal amounts of cell lysate protein into each immunoprecipitation by Ponceau red staining (data not shown). To further ensure that the result illustrated by Fig. 7A correctly identified the TNF-activated CREB kinase, the experiment was repeated independently three times, Western blots were scanned, and the activity in each enzyme reaction is reported as fold-activation relative to control. The data in Fig. 7B confirm that TNF induced significant activation of MSK1 and that such activation was reproducibly dependent on p38 MAPK activity.

**DISCUSSION**

CREB is a 43-kDa member of the CREB/CREM/ATF family of nuclear transcription factors that was first identified as a mediator of cyclic AMP-induced gene expression (60). Activation of CREB is mediated by phosphorylation of serine-133 (24), which augments CREB-induced gene transcription (22). CREB plays an important role in cell proliferation, differentiation, and adaptive responses. CREB is necessary for generation of the normal repertoire of T cell lineages, and the absence of CREB is associated with adult dwarfism and cardiac myopathy (5, 17, 66). Peptide hormones, growth factors, cytokines, neuronal activity, ultraviolet irradiation, and crosslinking of surface IgG activate CREB (7, 22, 33, 61, 67, 72). This diversity of signal initiation, and the different signaling systems through which these cellular stressors act, indicates that serine-133 in CREB may be a substrate for a multiplicity of protein kinases.

CREB phosphorylation is induced by mitogenic signaling pathways downstream of tyrosine kinase receptors, such as those for nerve growth factor and epidermal growth factor (13, 74). The mitogen-induced signaling pathway commences with stimulation of guanine nucleotide exchange factors, such as SOS, which activates Ras. Ras induces activation of the Raf serine-threonine kinase, an upstream activator of MAP kinase kinases (MEKs). Downstream targets of the MEKs are the MAP kinases ERK1 and ERK2, which can play a role in activation of ribosomal S6 kinase pp90RSK. Activated RSKs translocate from the cytoplasm into the nucleus, and there they phosphorylate CREB at serine-133 (73).

Exposure of cells to stress or cytokines can activate MAPK kinase 3/6 homologues, which in turn activate p38 MAPK signaling, an alternative pathway leading to CREB activation (14, 54, 67). Subsequently, p38 MAPK signaling can activate MAPKAP kinase 2, MAPKAP kinase 3, and MSK-1, which are CREB kinases (14, 54, 67, 75). Most insight into the significance of p38 MAPK to TNF function is based on the effects of bicyclic imidazole inhibitors, such as SB-203580, which at low concentrations, such as those employed in the present study, appears to specifically inhibit p38 MAPK function (12). These data suggest that p38 MAPK is involved in the upregulation of inflammation-related genes such as prostaglandin H synthase 2, collagenase-1, IL-6, IL-8, and TNF itself through effects on gene transcription, translation, or transcript stability (56).
Here, we show that TNF increases phosphorylation of nuclear CREB on serine-133 and augments CREB DNA binding and transactivation in endothelial cells. Specific pharmacological inhibitors identified signaling events that play a role in activation of CREB in HUVEC by TNF. ERK1, ERK2, and PI 3-kinase were excluded as mediators of TNF-induced CREB phosphorylation based on the inability of PD-98059, U-0126, and wortmannin to inhibit this event. Inhibition of p38 MAPK by SB-203580 completely abrogated TNF-induced activation of CREB. These results show that p38 MAPK mediates CREB phosphorylation in response to stimulation of HUVEC by TNF.

An in vitro kinase assay was used to identify the CREB kinase activated by TNF in HUVEC. In these experiments, ATF-1, which is highly homologous to CREB and can therefore serve as a substrate with which to assay the activity of CREB kinases (47), was used as a substrate. The experiments show that MSK-1 is a CREB kinase activated by TNF in HUVEC. Thus our observations show that a p38 MAPK/MSK1 signaling pathway can be used by TNF to communicate with the nucleus and, through transcriptional activation of CREB, may promote changes of gene expression in the endothelium.

The capacity of TNF to signal through TNFR-1 and TNFR-2 added an element of complexity to our studies. This is illustrated by observations showing that the TNF receptors are regulated independently of one another and that in some (31), but not all (48), cell types, both receptors can play a role in activation of NF-κB (31). Additional studies using TNF mutants that target one or the other receptor have suggested that TNFR-1 plays a predominant role in activation of NF-κB in cell types that express both receptors (9). Studies of wild-type and TNFR-1-deficient mouse fibroblasts have shown TNFR-1 to be the receptor that mediates cell adhesion and secondary factor release and proliferation, which are important to the fibroblast response to tissue injury and repair (46). In HUVEC, TNF-induced cell adhesion appears to be under the dominant control of the TNFR-1 (45). However, crosstalk between the receptors (41), ligand passing from TNFR-2 to TNFR-1 (68, 69), and the possibility that receptor function may be cell-type specific indicate how complex TNF function may be, particularly in vivo.

The present study has implicated p38 MAPK signaling in the induction of CREB activity by TNF. A previous study of wild-type and macrophages from TNFR-1- or TNFR-2-deficient mice suggested that the coincident expression of both receptors is necessary for activation of p38 MAPK by TNF (50). Stimulation of human airway smooth muscle cells with agonist antibodies directed against either receptor led to activation of p38 MAPK, showing that in this cell type, either receptor by itself may activate p38 MAPK (4). Treatment of KYM-1 human rhabdomyosarcoma cells KYM-1, human HeLa cervical epithelial cells, or HeLa cells engineered to overexpress TNFR-2 with TNF mutants that specifically bind one or the other TNF receptor, or with receptor-specific antibodies, showed that only TNFR-1 activated p38 MAPK in these cells (37). Thus either TNF receptor may activate a p38 MAPK signaling pathway and potentially could play a role in CREB activation, depending on the particular cell under study.

The demonstration that p38 MAPK is activated by TNF in HUVEC led us to investigate the possible roles of TNFR-1 and TNFR-2 in CREB activation in HUVEC. We evaluated which TNF receptor induced CREB activation by treating cells with neutralizing antibodies to each TNF receptor and then with TNF. An antibody directed against TNFR-1 blocked TNF-TNFR-1 activated p38 MAPK in these cells (37). Thus either TNF receptor may activate a p38 MAPK signaling pathway and potentially could play a role in CREB activation, depending on the particular cell under study.

Fig. 7. Signaling pathway used by TNF to activate CREB. A: an in vitro kinase assay, using ATF-1 as substrate, tested for activation of MAPKAP kinase 2, MAPKAP kinase 3, and MSK-1. The kinases were immunoprecipitated from lysates of HUVEC treated with vehicle or SB-203580 and then medium or TNF. A Western blot was probed with anti-phospho-CREB/ATF1, scanned, and the activity of each kinase, relative to control, is provided beneath the blot. B: the experiment described above was independently repeated 3 times, and the results were averaged and reported as fold-induction relative to control ± SD.
tion by promoting VEGF production (58) and by affecting expression of VEGF receptors (23, 51). TNF and VEGF cooperate in the formation of capillary-like structures during angiogenesis (38) and synergistically induce endothelial cell tissue factor, which can inhibit tumor growth through its clot-promoting procoagulant activity but promote angiogenesis by inducing production of growth regulatory molecules, including VEGF (1, 76). VEGF antagonizes endothelial cell death induced by TNF, and TNF inhibits the ability of VEGF to activate one of the two VEGF receptors, KDR, and endothelial cell proliferative responses dependent on KDR (27, 65). TNF (present study) and VEGF (47) both activate CREB in HUVEC, although they do so through different mechanisms. VEGF activates p38 MAPK/MSK-1 and protein kinase C/P90 RSK pathways downstream of the KDR receptor tyrosine kinase to activate CREB (47), whereas TNF induces a p38 MAPK/MSK-1 pathway downstream of TNFR-1. The interrelated functions of TNF and VEGF point to the need for the factors to affect expression of some common genes in endothelial cells. Thus the present study has identified a mechanism through which TNF can act on CREB, which is important to TNF action and the synergistic effects of TNF with other factors.

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