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

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Tumor necrosis factor activates CRE-binding protein through a p38 MAPK/MSK1 signaling pathway in endothelial cells. Am J Physiol Cell Physiol 286: C547–C555, 2004; 10.1152/ajpcell.00332.2002.—Tumor necrosis factor (TNF) promotes immunity and modulates cell viability, in part, by promoting alterations of cellular gene expression. The mechanisms through which TNF communicates with the nucleus and alters gene expression are incompletely understood. Incubation of human umbilical vein endothelial cells (HUVEC) with TNF induces phosphorylation of the CRE-binding protein (CREB) transcription factor on serine 133 and increases CREB DNA binding and transactivation. Dominant negative CREB, an antagonist antibody directed against the type 1 TNF receptor, or pharmacological inhibition of p38 MAPK signaling blocked TNF-induced CREB activation as determined by phosphorylation and gene reporter assays. From among the kinases that can activate CREB, we found that downstream of p38 MAPK, MSK1 is activated by TNF to promote CREB activation. These observations show that CREB is activated by TNF/TNFRI signaling through a p38 MAPK/MSK1 signaling pathway.

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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-κB 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 (Beverly, MA). Anti-MAPKAP kinase 2 and recombinant ATF-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPKAP kinase 3 and MSK-1 were from Upstate Biotechnology (Lake Placid, NY). The neutralizing antibodies directed against TNFR1 or TNFRII were from R&D Systems (Minneapolis, MN). The 3XCRE luciferase reporter plasmid was from Stratagene (La Jolla, CA). All inhibitors were from Calbiochem (San Diego, CA) or Sigma (St. Louis, MO).

Cell culture and Western blotting. HUVEC were grown on gelatin-coated tissue culture plates in endothelial cell growth medium (Clonetics, San Diego, CA) under 5% CO₂ at 37°C. For TNF stimulation, the cells were cultured in endothelial cell basal medium (Clonetics) that was supplemented with 1% bovine serum albumin. After 16 h, the cells were treated with various inhibitors as described in the figure legends and then stimulated with 1 nM TNF for 20 min. Cells were harvested into lysis buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate) that was supplemented with 1 mM PMSF, 0.15 units/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A and incubated on ice for 30 min, being vortexed every 10 min. Fifty micrograms from each sample was fractionated by SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% nonfat dried milk in PBST. Antibodies used to probe the Western blots were phospho-p38MAPK, p38 MAPK, pMAPK, MAPK, pCREB, and CREB. Proteins on the Western blots were detected using the enhanced chemiluminescent detection system.

In vitro kinase assays. Whole cell lysates were prepared from HUVEC treated with medium or TNF for 20 min. Protein A/G agarose was added and incubation continued for 1 h. After centrifugation, the pellets were washed with kinase assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 75 mM MgCl₂, 1 mM dithiothreitol), and this procedure was repeated twice. The washed pellet was incubated for 10 min at 37°C in 25 μl of kinase assay buffer modified to contain 50 μM ATP and 1 μg of recombinant AP-1F. 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 [γ³²P]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 poly dIdC in a final volume of 20 μl were incubated on ice for 10 min, and then 5 ng of end-labeled oligonucleotide was added to the reaction, which was continued for an additional 20 min. The reaction mixture was fractionated on a 5% native polyacrylamide gel (1.5 h, 180 V). The gel was dried and exposed to film. NF-xB DNA binding was assayed by EMSA as previously described (28). For gene reporter assays, cells were transiently cotransfected with RSV-CRE and the 3XCRE reporter plasmid. After 24 h, luciferase activity was assayed in cell lysates and normalized to β-gal expression.

Confocal microscopy. HUVEC incubated with vehicle or SB-203580 for 1 h and then with TNF for 20 min were fixed, permeated with 0.1% Triton X-100, and blocked with 2% bovine serum albumin. Anti-phosphoCREB was added, followed by a Texas red-conjugated sheep anti-rabbit secondary antibody. The nucleus was detected by staining with nuclear dye Syto 16. Excitation of the stains was performed on a Bio-Rad MRC 1024 Krypton/Argon laser confocal imaging system.

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 transfectsed 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
A lysates were then probed with anti-phospho MAPK (C). Western blots of cell stimulated with 1 nM TNF for various times at 37°C. As illustrated in Fig. 2C, antibodies that antagonize signaling through either TNFR-1 or TNFR-2 substantially diminished the capacity to induce NF-κB DNA binding in HUVEC. Thus TNF-R2 has a substantial role in mediating the capacity of TNF to induce NF-κB DNA binding but does not appear to play a significant role in the induction of CREB activation by TNF.

To characterize the signaling events important for CREB activation, HUVEC were stimulated with TNF and Western blots were probed with antibodies that specifically recognize the phosphorylated (activated) forms of 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'-TGCCGTCA-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.
HUVEC. Attempts to demonstrate immunoprecipitation of equal amounts of MSK1, MAPKAP2, and MAPKAP3 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 antisera, 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-induced CREB phosphorylation, showing that this receptor mediates CREB activation (Fig. 2A). Statistical evaluation of results from multiple experiments (Fig. 2B), in which the data were normalized to account for small variances in protein loading, show that TNFR-1 is the major receptor used by TNF to activate CREB. Consideration of the results in Fig. 2, A and B, together makes it difficult to entirely preclude a lesser role for TNFR-2 in induction of CREB activity in HUVEC by TNF. The possibility of crosstalk between signaling pathways activated by the receptors and the possibility that TNFR-2 may pass TNF to TNFR-1 suggest that the conservative conclusion reached insofar as the potential roles of the TNF receptors in CREB activation is most appropriate. However, our results do indicate that TNFR-1 plays the predominant role in CREB activation in HUVEC. TNF has profound effects on the endothelium and can promote or inhibit the activity of endothelial specific mitogens such as VEGF. TNF is involved in wound repair and inflammation, processes in which VEGF is elaborated and important (18, 19, 70). TNF modulates VEGF func-

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.

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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 (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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