Inhibition of Tumor Necrosis Factor Signal Transduction in Endothelial Cells by Dimethylaminopurine*

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Tumor necrosis factor (TNF) promotes diverse responses in endothelial cells that are important to the host response to infections and malignancies; however, less is known of the postreceptor events important to TNF action in endothelial cells than in many other cell types. Since phosphorylation cascades are implicated in cytokine signaling, the effects of the protein kinase inhibitor dimethylaminopurine (DMAP) on TNF action in bovine aortic endothelial cells (BAEC) were investigated. In BAEC, TNF promotes phosphorylation of eukaryotic initiation factor 4E (eIF-4E), c-Jun N-terminal kinase (JNK) and ceramide-activated protein kinase, which share homologies with one another, whereas the intracellular elements in the TNF signal transduction cascade, most particularly proteins that bind directly to either TNF receptor, are less well characterized. TNF initiates its diverse actions by binding to either of two receptors (5, 6). The extracellular domains of the receptors share homologies with one another, whereas the intracellular domains do not; thus, it is not surprising that the receptors induce distinct responses (7–14). Neither TNF receptor contains intrinsic protein tyrosine kinase activity or any recognizable motif, which suggests a biochemical activity (5, 6). Receptors that do not contain enzyme activity interact with accessory proteins, which couple the receptor to signaling pathways (15, 16). For this reason, recent studies from a number of laboratories, including our own, have been directed at identifying proximal elements in the TNF signal transduction cascade, most particularly proteins that bind directly to either TNF receptor and may promote cellular responses (17–24).

As important as understanding how TNF receptors couple to effector mechanisms is characterization of downstream elements in the signaling cascade that promote responses. As with other hormones, the phosphorylation of specific target proteins resulting in the production of mediators, alterations of gene expression, and changes of cell function and/or viability is associated with TNF action in some cell types (25), although the endothelial cell has not been particularly well investigated. In the present study, the protein kinase inhibitor dimethylaminopurine (DMAP) (26–29) was used to study TNF signaling in bovine aortic endothelial cells (BAEC), as it displays a unique scope of action that distinguishes it from other protein kinase inhibitors. DMAP induces premature mitosis in mammalian cells (28) and inhibits a protein kinase that supports the maturation of starfish oocytes (29). An analog of DMAP, 2-aminopurine, blocks the induction of human β-interferon and

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- ‡§The abbreviations used are: TNF, tumor necrosis factor; DMAP, dimethylaminopurine; BAEC, bovine aortic endothelial cell(s); HUVEC, human umbilical vein endothelial cells; eIF-4E, eukaryotic initiation factor 4E; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; CAP, ceramide-activated protein; EF-2, elongation factor-2.
c-Fos and c-Myc expression by virus in various cell lines (30). Here, we report that TNF induces a distinct signal transduction cascade in BAEC that can be distinguished from that employed by histamine and is inhibited selectively by DMAP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human TNF-α (5 × 10̇ units/mg) was a gift from Genentech. [γ-32P]dCTP (3000 Ci/mmol) and carrier-free Na125I (5 ̃Ci/mCi) were from Amersham. Sephadex G-25 (fine) and oligolabeling kits were from Pharmacia. BA-S supported nicotinucleosome membranes were from Schleicher & Schuell. DMAP and cycloheximide were from Sigma. Other materials were from standard suppliers.

**Cell Culture**—Cells were grown on gelatin-coated dishes. To prepare these, a 24-well dish was solution was added to dishes, which were allowed to stand at room temperature for 1 h. The gelatin solution was then removed by aspiration. BAEC were maintained in Medium 199 supplemented with 20% heat-inactivated fetal bovine serum, 20 mM Hepes, pH 7.4, 10% dextran sulfate, and 40 g/ml salmon sperm DNA and 2 g/ml of lysis buffer (20 mM Tris-HCl, pH 8.8, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate, 1% Nonidet P-40, 0.15 mM/ml aprotinin, 0.01 mg/ml leupeptin). The mixture was gently vortexed, incubated on ice for 20 min, and centrifuged at 14,000 rpm for 20 min at 4°C. 100 mg/ml of the supernatant was incubated with 10 μl of glutathione-S-transferase-c-Jun for 4 h at 4°C. The beads were pelleted and washed twice with glutathione-S-transferase-lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40) and once with distilled water. Finally, the beads were resuspended in 20 μl of kinase assay buffer (20 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 2 mM MgCl2, 2 mM MnCl2, 5 mM NaF, 1 mM sodium orthovanadate) that contained 1 μCi of [γ-32P]ATP and incubated for 20 min at 30°C. After addition of 2 × Laemmli sample buffer and protein fractionation on 12% polyacrylamide gels, Jun kinase activity was evaluated by autoradiography and phosphoimage analysis.

**Isolation of RNA and Northern Blot Analysis**—Total cellular RNA was extracted from 5 × 10̇ cells into 4 μl guanidinium isothiocyanate, 0.1% sodium lauryl sarcosine, 0.5% sodium dodecyl sulfate, and 40 mM EDTA in cassettes containing Dupont IODO-GEN. 125I-TNF (specific activity 60 Ci/g) was separated from TNF by using oligolabeling kit. Hybridizations were carried out during an overnight incubation at 42°C in prehybridization solution containing 100 g/ml salmon sperm DNA and 2 × 10̇ g/ml of the probe for c-Jun (a gift from Dr. Ethan Dmitrovsky, Memorial Sloan-Kettering Cancer Center) or glyceraldehyde-3-phosphate dehydrogenase. The filters were washed twice with 0.1 × SSC, 0.1% SDS for 30 min at 55–60°C. Autoradiography was conducted at −70°C for 2–5 days.

**Prostaglandin Production**—Confluent BAEC in 35-mm Petri dishes were washed three times with Hank’s balanced salt solution. Cells were treated in the absence or presence of 1 μM DMAP for 15 min before addition of 1 nM TNF or 100 μM histamine. After incubation at 37°C for 16 h with TNF or for 5 min with histamine, supernatants were collected and assayed by enzyme immunoassay for 6-keto-prostaglandin F1α, which is the stable degradation product of prostaglandin F1a, as described (36, 37).

**Cellular Cytotoxicity**—BAEC were incubated with TNF, cycloheximide, or TNF plus cycloheximide in the absence or presence of DMAP for 6 h at 37°C. Cells were washed twice with phosphate-buffered saline, detached from the culture plates using 0.05% trypsin, 0.5 mM EDTA, and counted in a Coulter counter. Cell viability was verified by Trypan blue exclusion.

**Binding of 125I-TNF to BAEC**—125I-TNF was coupled to TNF by using IODO-GEN. 125I-TNF (specific activity 60 Ci/g) was separated from Na125I by chromatography on Sephadex G-25 (fine). 125I-TNF was greater than 95% precipitable in 5% (w/v) trichloroacetic acid and retained greater than 80% of the antiproliferative activity of unlabeled TNF on the ME-180 line of human cervical carcinoma cells. Confluent BAEC in six-well plates were incubated for 16 h at 4°C in Hank’s balanced salt solution, 1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 1 μM 125I-TNF in the absence or presence of 500 nM TNF. Cells were washed twice with ice-cold Hank’s balanced salt solution, solubilized into 1 μl NaOH/1% SDS and assayed for specific binding. The difference in radioactive uptake with and without unlabeled TNF, in a γ counter.

**RESULTS**

We previously demonstrated that TNF rapidly (within minutes) promotes the phosphorylation of an mRNA cap-binding protein, eIF-4E, in several cell types that are important in vitro models of TNF action (38). Phosphorylation promotes interac-

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activation was inhibited by pretreatment of BAEC with 1 mM DMAP for 10 min and eIF-4E was isolated as described under "Experimental Procedures." The data shown are representative of observations from three independent experiments. Top, eIF-4E phosphorylation was evaluated after SDS-PAGE and autoradiography. Bottom, the results were analyzed with a densitometer and are presented in arbitrary scanning units.

The complexes produced interact with ribosomes where translation initiates. Thus, by activating eIF-4E, TNF may alter the pattern of protein synthesis and the function of cells. In the present study, we evaluated the effects of DMAP on this early cellular response to TNF. To accomplish this, the ATP pools of BAEC were equilibrated with 32P during a 45-min incubation at 37 °C and then stimulated with 1 nM TNF for 15 min at 37 °C. The cells were then stimulated with 1 nM TNF for 10 min and eIF-4E was isolated as described under "Experimental Procedures." The data shown are representative of observations from three independent experiments. Top, eIF-4E phosphorylation was evaluated after SDS-PAGE and autoradiography. Bottom, the results were analyzed with a densitometer and are presented in arbitrary scanning units.

whether such activation could be inhibited by DMAP, these cells were treated with vehicle or DMAP and then stimulated with 10 nM TNF. JNK activity was then measured by a solid phase kinase assay using glutathione S-transferase-c-Jun as substrate. As shown in Fig. 3, TNF augmented JNK kinase activity in BAEC and this effect was substantially inhibited by 1 mM DMAP.

We (45) and others (see Ref. 46 and references therein) have shown that TNF promotes expression of early response genes in diverse cell lines. Experiments were performed to determine whether TNF promotes such gene expression in BAEC and whether DMAP might suppress such a response. Fig. 4 shows that incubation of BAEC with TNF augmented expression of Jun-b within 30–60 min. Pretreatment of cells with 1 mM DMAP for 15 min abrogated the ability of TNF to promote Jun-b expression.

Prostacyclin is a labile prostaglandin, which inhibits platelet aggregation and serotonin release and also promotes vasodilation (47, 48). One of the important responses induced by TNF in the endothelium is production of prostacyclin (49, 50). For this reason, we tested whether DMAP affects this event. As shown in Fig. 5A, treatment with TNF for 16 h induces a 4-fold increase in prostaglandin production by BAEC. By itself, incubation with DMAP had no effect on the production of prostacyclin. However, coincubation with DMAP (1 mM) abrogated the ability of 1 nM TNF to elicit this response. In a separate experiment, histamine (100 μM) promoted a 4-fold increase in prostaglandin synthesis by BAEC, but this process was not inhibited by DMAP (Fig. 5B). The ability of DMAP to affect signaling induced by TNF, but not by histamine, demonstrates that the effects of DMAP on responses to TNF are specific.

These observations also suggest that TNF and histamine do not act through identical postreceptor signaling pathways in BAEC. Consistent with this suggestion are observations show-
ing that prostacyclin induction by histamine reaches a maximal level within minutes (51), whereas induction following treatment of endothelial cells with TNF initiates within minutes (52), but then continues to increase for at least 16 h (49). Additionally, in contrast to TNF, histamine is unable to promote eIF-4E phosphorylation (data not shown), but does induce phosphorylation of elongation factor-2 (EF-2) (53).

This latter observation led us to test whether DMAP could suppress the ability of histamine to promote EF-2 phosphorylation. To accomplish this, BAEC were treated for various times with histamine (0.1 mM), DMAP (1 mM), or histamine together with DMAP. Proteins in cell lysates were then fractionated by SDS-PAGE and transferred to nitrocellulose which was probed with an antibody that recognizes phosphorylated, but not non-phosphorylated, EF-2. This led to the surprising observation that DMAP as well as histamine promoted a time-dependent increase of EF-2 phosphorylation (Fig. 6, bottom). To ensure that this observation was not unique to BAEC, the experiment was repeated with HUVEC with similar results (Fig. 6, top). In neither BAEC nor HUVEC did DMAP attenuate EF-2 phosphorylation induced by histamine; rather, the combination of the two reagents further increased EF-2 phosphorylation. These observations reinforce the view that DMAP selectively acts on components of the TNF signal transduction pathway and also indicate that this reagent may be useful in promoting some cellular responses to histamine by augmenting postreceptor events.

TNF is variously reported to have no effect on the viability of endothelial cells or to induce cytosasis or apoptosis (54–56). The response to TNF may depend on the number of times that endothelial cell cultures have been passed (56), or other as yet unidentified variables. In our hands, TNF, at concentrations up to 1 nM and over a period of 6 h, did not elicit cytotoxicity or inhibit the proliferation of BAEC (data not shown). This observation was not unexpected as many non-transformed cells contain salvage proteins that protect them from TNF-induced cytotoxicity. Consistent with this is the demonstration that inhibition of protein synthesis sensitizes cells to TNF-induced cytotoxicity, presumptively through loss of protective proteins (54, 57). We now report that whereas TNF or cycloheximide, an inhibitor of protein synthesis, by themselves did not induce a cytotoxic response from BAEC, coinubcation of cells with both...
agents did induce cytotoxicity. This effect was manifest within 1 h, and a near complete loss of cell viability was observed within 6 h (90% cell kill). These observations permitted us to determine whether DMAP was capable of inhibiting the cytotoxic response elicited from BAEC by TNF and cycloheximide. As illustrated in Fig. 7, DMAP substantially attenuated TNF/cycloheximide-promoted cytotoxicity in BAEC and augmented cell viability from 14% to about 50%.

The type 1 TNF receptor associates with a protein serine kinase, the TNF receptor-associated kinase, in various cells (17–18). In additional experiments (data not shown), we found that a protein kinase associates with type 1 TNF receptor in BAEC and that DMAP inhibited this activity. However, we were unable to demonstrate that TNF could stimulate the TNF receptor associated kinase in BAEC, apparently due to high basal activity.

To determine whether the effects of DMAP on TNF signaling result from alterations of receptor expression or changes in post-receptor activities, the effect of various concentrations of DMAP on the specific binding of 125I-TNF to BAEC was evaluated. In the experiment illustrated by Fig. 8, cells were incubated with DMAP (0.05–1 mM) for 6 h before the specific binding of 125I-TNF to the cell cultures was assayed. DMAP did not significantly affect 125I-TNF binding by BAEC, which suggests that its effects result from actions at postreceptor sites.

**DISCUSSION**

The mechanisms that transduce TNF binding into responses are incompletely defined. In a recent, comprehensive study of protein phosphorylation in fibroblasts, TNF was shown to induce alterations in the phosphorylation state of a diverse group of proteins, increasing the phosphorylation of some and decreasing the phosphorylation of others (58). Heat shock, epidermal growth factor, bradykinin, mellitin, and activators of protein kinase C and protein kinase A induce distinct patterns of protein phosphorylation, none of which are similar to the phosphorylations induced by TNF and interleukin-1, which share many common activities (58). These observations suggest that these cytokines can activate unique signaling mechanisms. Consistent with this conclusion are observations showing that in addition to activating protein kinase C (59), protein kinase A (60), and mitogen-activated protein kinases (61), which are implicated in the actions of many hormones, TNF also activates a group of novel kinases, including the JNK kinases (44), a ceramide-activated protein kinase (41, 42), and protein kinase A (60), and mitogen-activated protein kinases (61), which are implicated in the actions of many hormones. TNF also activates a group of novel kinases, including the JNK kinases (44), a ceramide-activated protein kinase (41, 42), and protein kinase A (60), and mitogen-activated protein kinases (61), which are implicated in the actions of many hormones. TNF also activates a group of novel kinases, including the JNK kinases (44), a ceramide-activated protein kinase (41, 42), and

**FIG. 7. Effect of DMAP on cytotoxicity.** BAEC were treated for 6 h at 37°C with 1 mM DMAP in the absence or presence of TNF (1 nM), cycloheximide (10 μg/ml), or TNF (1 nM) + cycloheximide (10 μg/ml), and cell number was assayed using a Coulter counter. Data are shown as the mean ± S.E., n = 3.

**FIG. 8. Effect of DMAP on specific binding of 125I-TNF to BAEC.** BAEC were incubated in the absence or presence of various concentrations of DMAP for 6 h. The binding of 125I-TNF to the cells was then assayed as described under “Experimental Procedures.” Data are shown as mean ± S.E., n = 3.

TNF receptor-associated serine/threonine kinases (17–19).

Studies with inhibitors also point to a role for phosphorylation/dephosphorylation reactions in TNF action. Staurosporine, a protein kinase inhibitor, sensitizes tumor cell lines to TNF-mediated cytotoxicity and also to TNF-promoted activation of phospholipases C and D and the transcription factor NF-κB (62). These effects are apparently mediated through actions on a unique intracellular target, as other protein kinase inhibitors do not produce similar synergy with TNF. Okadaic acid, a serine and threonine phosphatase inhibitor, mimics the changes of protein phosphorylation and gene transcription induced by TNF in fibroblasts (63), but inhibits TNF-mediated cytotoxicity in transformed cells (64), an effect also observed with the tyrosine phosphatase inhibitor orthovanadate (64). The observations summarized above suggest that a tightly regulated network of cellular kinases and phosphatases affects TNF action through both positive and negative regulation.

The present study tested whether protein kinase activity is associated with TNF signaling in endothelial cells. To accomplish this we characterized signaling in BAEC and showed that TNF enhances the phosphorylation of eIF-4E, activates JNK and CAP kinases, augments Jun-b expression, elevates the production and secretion of prostacyclin, and, when protein synthesis is inhibited, induces cytotoxicity. The protein kinase inhibitor DMAP was tested for its effect on these events and abrogated or significantly attenuated each.

The cellular response to TNF and the effects of DMAP on the cellular response to TNF were specific. A number of observations support these conclusions. First, TNF, but not histamine, induces phosphorylation of eIF-4E, indicating that there are differences in the kinase cascades induced by these reagents. Second, TNF and histamine induce prostacyclin production; however, DMAP inhibits induction of prostacyclin by TNF but not by histamine. Third, histamine, but not TNF, promotes phosphorylation of EF-2. This phosphorylation was not inhibited by DMAP; in fact, this unique reagent by itself promoted EF-2 phosphorylation. Why DMAP increases EF-2 phosphorylation is unclear. Since EF-2 is a specific substrate for a novel calcium/calmodulin-dependent protein kinase (65), one possible interpretation of this effect is that DMAP increases intracellular calcium; however, this remains to be tested as do the effects of DMAP on cellular protein synthesis.

Regardless of its mechanism of action, the observations presented in this report show that DMAP may be useful in delineating the TNF signaling pathway. The presence of unique elements in the TNF signal transduction cascade, which are
amenable to selective inhibition by reagents such as DMAP, may prove to be useful targets in developing therapies for pathologies associated with cytokine elaboration. Additionally, DMAP may prove useful in investigations of histamine action and histamine signal transduction in the endothelium.

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