Inhibition of endocytosis exacerbates TNF-α-induced endothelial dysfunction via enhanced JNK and p38 activation

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Inhibition of endocytosis exacerbates TNF-α-induced endothelial dysfunction via enhanced JNK and p38 activation. Am J Physiol Heart Circ Physiol 306: H1154–H1163, 2014. First published February 21, 2014; doi:10.1152/ajpheart.00885.2013.—Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that causes endothelial dysfunction. Endocytosis of TNF-α receptors (TNFR) precedes endosomal reactive oxygen species (ROS) production, which is required for NF-κB activation in vascular smooth muscle cells. It is unknown how endocytosis of TNFRs impacts signaling in endothelial cells. We hypothesized that TNF-α-induced endothelial dysfunction is induced by both endosomal and cell surface events, including NF-κB and mitogen-activated protein kinases (MAPKs) activation, and endocytosis of the TNFR modifies signaling. Mesenteric artery segments from C57BL/6 mice were treated with TNF-α (10 ng/ml) for 22 h in tissue culture, with or without signaling inhibitors (dynasore for endocytosis, SP600125 for JNK, SB203580 for p38, U0126 for ERK), and vascular function was assessed. Endothelium-dependent relaxation to acetylcholine (ACh) was impaired by TNF-α, and dynasore exacerbated this, whereas JNK or p38 inhibition prevented these effects. In cultured endothelial cells from murine mesenteric arteries, dynasore potentiated JNK and p38 but not ERK phosphorylation and promoted cell death. NF-κB activation by TNF-α was decreased by dynasore. JNK inhibition dramatically increased both the magnitude and duration of TNF-α-induced NF-κB activation and potentiated intercellular adhesion molecule-1 (ICAM-1) activation. Dynasore still inhibited NF-κB activation in the presence of SP600125. Thus TNF-α-induced endothelial dysfunction is both JNK and p38 dependent. Endocytosis modulates the balance of NF-κB and MAPK signaling, and inhibition of NF-κB activation by JNK limits this pro-proliferative signal, which may contribute to endothelial cell death in response to TNF-α.

tumor necrosis factor-α; endocytosis; endothelial dysfunction; JNK; p38 kinase; NF-κB

TUMOR NECROSIS FACTOR-α (TNF-α) is a critical regulator of the inflammatory response. TNF-α levels are elevated in inflammatory and infectious conditions (11, 27, 41, 48, 60), including the metabolic syndrome characterized by obesity, diabetes (24, 57), hypertension (19), and ischemic heart diseases (56). Thus TNF-α is an important risk factor for cardiovascular diseases for which endothelial dysfunction is a hallmark (3). There is accumulating evidence that TNF-α plays a fundamental role in the development of endothelial injury (6) and induces expression of inflammatory cell-recruiting chemokines (12) and apoptosis (49). In contrast, TNF-α primarily promotes proliferation and migration of vascular smooth muscle cells (20, 45).

TNF-α binds to two distinct receptor subtypes, TNF-α receptor 1 (TNFR1) and TNFR2. Previous analysis of TNFR signaling in endothelial cells has demonstrated a strong TNFR1 dominance in mouse pulmonary microvascular endothelial cells (5) and in human umbilical vein endothelial cells (HUVECs) (42). TNFR1 stimulates two seemingly opposing pathways: nuclear factor-kappa B (NF-κB) resulting in cell survival and proliferation, and c-Jun NH2-terminal kinase (JNK) signaling pathways leading predominantly to cell death.

Endocytosis is required for both signaling and recycling of TNFRs, and endocytosis of TNFR1 plays a regulatory role in signal transduction (51, 52). We have demonstrated previously that endosomes are the site of reactive oxygen species (ROS) production in response to TNF-α, and ROS at this site are required for NF-κB activation and vascular smooth muscle cell (VSMC) proliferation (35, 37, 38). However, it is unknown how receptor endocytosis impacts the balance of pro-proliferative vs. pro-apoptotic effects of TNF-α in endothelial cells. Global regulation of the endocytic process can modify responsiveness to multiple cytokine receptors (44), and specific components of the endocytic machinery are selectively regulated. Dynamin is essential for both clathrin-mediated endocytosis and for internalization of caveolae. It acts to sever the neck of membrane invaginations and pinch off endocytic vesicles, separating them from the plasma membrane (23, 34, 52). Dynamin is regulated by phophatidylinositol and by its binding partner amphiphysin. Cyclin-dependent kinase 5 (Cdk5) phospholylates and regulates both dynamin 1 and amphiphysin 1 (54). Dynamin controls the rate of maturation of clathrin-coated pits and determines whether forming pits mature into endocytic vesicles (32).

In addition to NF-κB, TNF-α also activates multiple mitogen-activated protein kinases (MAPKs), including JNK, p38 kinase, and extracellular signal-regulated kinase (ERK) (1). These MAPKs mediate multiple downstream TNF-α effects, including apoptosis, proliferation, or differentiation. It has been shown using various TNFR2 mutants that TNFR2 internalization induces JNK-mediated mouse lung endothelial cell apoptosis (26). However, it is unclear whether the transition of endogenous TNFRs, including TNFR1 and TNFR2, from the plasma membrane to endosomes modulates MAPK activation. Although NF-κB is known to affect JNK activity (13, 55), to our knowledge, JNK has not been previously demonstrated to modulate NF-κB activation by TNF-α.

Although the signaling literature tends to focus on cellular events occurring at relatively short intervals (minutes to hours) after TNF-α exposure, physiological cytokine exposure, as in the setting of sepsis or a systemic inflammatory response, is sustained over hours to days. We characterized the impact of endocytosis inhibition on endothelial dysfunction induced by sustained exposure to TNF-α in isolated mesenteric resistance arteries from mice. To complement these experiments, we explored the ability of receptor endocytosis to modify MAPK phosphorylation and NF-κB activation in cultured endothelial
cells from the same vessel. We hypothesized that TNF-α-induced impairment of endothelium-dependent vascular relaxation is related to both NF-κB and MAPK activation and that activation is related to both NF-κB-induced impairment of endothelium-dependent vascular relaxation and MAPK activation. Calcium Cl₃·2H₂O, 14.9 NaHCO₃, 5.6 glucose, and 0.03 EDTA. Mesenteric rings were incubated in Dulbecco’s modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS). Cultures were maintained in selective endothelial cell media as supplied by the vendor. Adenoviral-mediated gene transfer. Adenoviruses were obtained from Gene Transfer Vector Core at the University of Iowa. Control viruses (eGFP) or the dynamin dominant negative mutant (K44A) were added to endothelial cells (80% confluence, 1–10 MOI) in endothelial cell media containing 5% fetal bovine serum (FBS). Experiments were performed after 48 h. Detection of endocytosis. Detection of endocytosis was performed using the fluorescein reagent Texas Red conjugated to 10,000 MW Dextran (D-1863, Life Technologies, Grand Island, NY). Cells were grown on chamber slides coated with 0.2% gelatin, then incubated with Dextran (D-1863, Life Technologies, Grand Island, NY). Cultures were maintained in selective endothelial cell media as supplied by the vendor. Adenoviral-mediated gene transfer. Adenoviruses were obtained from Gene Transfer Vector Core at the University of Iowa. Control viruses (eGFP) or the dynamin dominant negative mutant (K44A) were added to endothelial cells (80% confluence, 1–10 MOI) in endothelial cell media containing 5% fetal bovine serum (FBS). Experiments were performed after 48 h. Detection of endocytosis. Detection of endocytosis was performed using the fluorescein reagent Texas Red conjugated to 10,000 MW Dextran (D-1863, Life Technologies, Grand Island, NY). Cells were grown on chamber slides coated with 0.2% gelatin, then incubated with adenovirus for 2 days or dynasore (15 μM) for 30 min, and then exposed to 100 μg/ml Dextran Texas Red plus TNF-α (10 ng/ml) for 30 min at 37°C. Cells were fixed in 3.7% formaldehyde and coverslides mounted with ProLong Gold anti-fade reagent (Life Technologies). The signals were imaged by fluorescence confocal microscopy using excitation at 595 nm and emission at 615 nm. Quantification of endosome number was performed using ImageJ software. Materials and methods. Reagents. Dynasore was obtained from Enzo Life Sciences (Farmingdale, NY). SP600125, SB203580, and U0126 were purchased from Cell Signaling Technology (Danvers, MA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cultured cells. Primary endothelial cells from C57BL/6 mice mesenteric arteries were obtained from Cell Biologics (Chicago, IL). Cultures were maintained in selective endothelial cell media as supplied by the vendor.

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Animals. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), 12–15 wk of age, were used in this study. All procedures were performed in accordance with the Guide for the Care and Use of Animals, approved by the Vanderbilt University Institutional Animal Care and Use Committee. The animals were housed on a 12-h light/dark cycle and fed a standard chow diet with water ad libitum.

Isolation of mesenteric arteries and functional studies. First-order mesenteric resistance arteries (~100 μM ID) were excised, cleaned of fat and connective tissues, and cut into 2-mm-length rings in an ice-cold physiological salt solution (PSS) consisting of the following (in mM): 130 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.18 MgSO₄·7H₂O, 1.56 CaCl₂·2H₂O, 14.9 NaHCO₃, 5.6 glucose, and 0.03 EDTA. Mesenteric rings were incubated in Dulbecco’s modified Eagle medium (DMEM) containing TNF-α (10 ng/ml, 22 h). Inhibitors (dynasore, 15 μM; SP600125, 3 μM; SB203580, 1 μM; U0126, 1 μM) were applied 30 min before TNF-α. Rings were subsequently mounted in wire myographs (Danish Myo Technology A/S, Aarhus, Denmark) containing warmed (37°C), oxygenated (95% O₂/5% CO₂) PSS and allowed to equilibrate for at least 45 min under a passive force of 2 mN. Arterial integrity was assessed by stimulation of vessels with 120 mM KCl and, after contraction, reached a plateau, and the rings were washed. Subsequently, the rings were stimulated with phenylephrine (PE; 10⁻⁶ M), followed by relaxation with acetylcholine (ACH; 10⁻⁵ M). More than an 80% relaxation response to ACH was taken as evidence of an intact endothelium. Contractile responses were assessed by cumulative exposure to PE (10⁻⁹ to 3 × 10⁻⁵ M). Endothelium-dependent relaxation was performed on PE-contracted (10⁻⁶ M) rings by cumulative addition of ACH (10⁻⁸ to 10⁻⁴ M), and endothelium-independent relaxation was tested using sodium nitroprusside (SNP; 10⁻⁹ to 10⁻⁵ M).

Western blot analysis. Protein extracts (40–60 μg) were separated by electrophoresis on a polyacrylamide gel (10%) and transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% skimmilk in Tris-buffered saline solution with TWEEN 20 (0.1%) for 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C. Antibodies were as follows: p-JNK, JNK, p-p38, p38, p-ERK, ERK, p-eNOS (S1177), eNOS, cleaved PARP (Cell Signaling Technology, Danvers, MA), iNOS (BD Biosciences, San Jose, CA), ICAM-1 (Developmental Studies Hybridoma Bank, Iowa City, IA), and Tubulin (Vanderbilt Antibody Core, Nashville, TN). After incubation with fluorescent secondary antibodies, signals were developed using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE) and quantified densitometrically. Results were normalized to α-tubulin or indicated protein and expressed as arbitrary units.

Measurement of nitric oxide. Cells grown on 96-well plates were treated with TNF-α (10 ng/ml) with or without dynasore (15 μM) for 24 h. Nitric oxide (NO) production was measured using 4,5-diaminofluorescein diacetate (DAF-2; Cayman Chemical, Ann Arbor, MI). Cells were incubated with DAF-2 in PBS for 30 min at room temperature and then replaced with PBS. Fluorescence was measured using excitation at 485 nm and emission at 520 nm in a microplate reader (FLUOstar Omega, BMG Labtech) and normalized to protein concentration (BCA protein assay).

Sulfurhodamine B assay. Cells grown on 96-well plates were treated with TNF-α (10 ng/ml) with or without dynasore (15 μM) and for 24 h. The sulfurhodamine B (SRB) assay was performed as previously reported (59). Briefly, cells were fixed with 5% cold trichloroacetic acid for 1 h at 4°C and washed with water followed by air dry at room temperature. The cells were then stained with 0.057% SRB solution for 30 min and rinsed with 1% acetic acid. After the plate was dried, 10 mM Tris base (pH 10.5) was added, and then the OD was measured at 510 nm in a microplate reader (FLUOstar Omega, BMG Labtech).

NF-κB activity. NF-κB-mediated transcriptional induction was assessed by infection of endothelial cells with replication-deficient adenovirus, containing a luciferase reporter gene driven by NF-κB transcriptional activation (38). Cells were infected for 40 h followed by exposure to TNF-α (10 ng/ml) in serum-free DMEM for 6 h. In experiments requiring longer TNF-α exposure (24 and 48 h), endothelial cell media with 5% FBS was used. Luciferase activity (relative light units) was measured in reporter lysis buffer, according to the protocol of the manufacturer (Promega, Madison, WI), and normalized to protein concentration (BCA protein assay).

Statistical analysis. Values are means ± SE, and n represents the number of animals used in the experiments or of independently performed experiments in cultured cells.

Contractions were recorded as changes in tension (mN) from baseline, expressed as a percentage of the response to 120 mM KCl. Relaxation was expressed as a percentage of the contraction produced by PE in each ring. Return to the baseline tension before addition of PE was considered to be 100% relaxation. Concentration-response curves were fitted using a nonlinear, interactive fitting program (Graph Pad Prism 5.0; GraphPad Software, San Diego, CA), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or Eₘₐₓ) and EC₅₀ (molar concentration of agonist producing 50% of the maximum response) or pD₂ (−log EC₅₀). Statistical differences were calculated by Student’s t-test or one-way ANOVA. Post hoc testing was performed using Newman-Keuls analysis to compare all of the groups. A P value of <0.05 was considered to be statistically significant.

Results. To selectively inhibit endocytosis, we compared recombinant dominant-negative dynamin (DynK44A) with a soluble dynamin inhibitor, dynasore. A point mutation in the nucleotide-binding site (K44A) interferes with endogenous dynamin function and prevents vesicle internalization (10). DynK44A effectively blocked TNF-α-induced uptake of dextran-conju-
gated Texas Red in endothelial cells (Fig. 1A). Dynasore (15 μM), a cell-permeable small molecule, is a noncompetitive inhibitor of dynamin GTPase activity that also blocks dynamin-dependent endocytosis (34). It is widely used as a selective inhibitor of the endocytic process (7, 21). Dynasore was equally as efficacious as DynK44A at preventing the internalization of dextran-conjugated Texas Red in cultured endothelial cells (Fig. 1B).

Effects of endocytosis and MAPKs on endothelial dysfunction induced by TNF-α. In endothelium-intact mesenteric arteries, TNF-α and dynasore, alone or in combination, did not change contractile responses to PE (Fig. 2A). Prolonged TNF-α exposure reduced sensitivity to the endothelial vasodilator ACh, but did not reduce the maximal relaxation response. By itself, dynasore had no effect on endothelium-dependent relaxation to ACh. However, dynasore dramatically exacerbated the endothelial dysfunction induced by TNF-α, including a large reduction in the maximal response (Fig. 2B). Endothelium-independent relaxation to SNP was not affected by TNF-α or dynasore plus TNF-α (Fig. 2C).

To test the hypothesis that TNF-α impairs endothelium-dependent relaxation through MAPK activation, we assessed ACh-induced relaxation in mesenteric arteries incubated with TNF-α in the presence of JNK, p38, or ERK inhibitors (SP600125, SB203580, and U0126, respectively). Both SP600125 and SB203580 prevented impairment of relaxation responses to ACh by TNF-α (Fig. 3, A and B). However, ERK inhibition (U0126) reduced the relaxation response itself and did not alter

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**Fig. 1.** Inhibition of the dynamin function prevents dextran endocytosis. A: endothelial cells were incubated with dextran-conjugated Texas red and TNF-α for 30 min after infection of eGFP control or DynK44A adenovirus (3 MOI) for 48 h. B: after incubation with DMSO or dynasore (15 μM) for 30 min, endothelial cells were incubated with dextran-conjugated Texas red and TNF-α for 30 min. The confocal images are representative of three independent experiments. Quantification of endosome number revealed a dramatic and comparable decrease in endocytosis was induced by DynK44A or dynasore. *Significant difference compared with eGFP or DMSO (P < 0.05).

**Fig. 2.** TNF-α impairs endothelium-dependent relaxation in an endocytosis-dependent manner in mesenteric arteries from mice. A: contraction to PE was measured in rings treated with vehicle (DMSO, control; n = 5), TNF-α (n = 6), dynasore (n = 3), and dynasore plus TNF-α (n = 3) for 22 h. B: relaxation to ACh was impaired by TNF-α, and this effect was exacerbated by dynasore (n = 6 to 7). C: relaxation to SNP was unaffected by TNF-α with or without dynasore (n = 6 to 9). *Significant difference of control vs. TNF-α for pD2 (P < 0.05). †Significant difference compared with TNF-α for Emax (P < 0.05).
the impact of TNF-α on relaxation (Fig. 3C). To discern whether the adverse effect of dynasore plus TNF-α on endothelial function is MAPK dependent, we tested relaxation responses to ACh following incubation in TNF-α and dynasore plus SP600125 or SB203580. These inhibitors fully protected against the severe impairment of relaxation to ACh induced by the combination of TNF-α and dynasore (Fig. 4). These data suggest that the exacerbation of endothelial dysfunction induced by TNF-α in the presence of the endocytosis inhibitor might be associated with enhanced JNK and/or p38 activation.

Activation of JNK and p38 in endothelial cells. The impact of TNF-α and TNFR endocytosis on MAPK activation was further investigated in cultured mesenteric arterial endothelial cells. JNK, p38, and ERK were all more phosphorylated following exposure to TNF-α for 24 h, and the effects on JNK and p38, but not ERK, were potentiated by dynasore (Fig. 5). These results suggest that TNFR-mediated JNK and p38 activation occur at the plasma membrane and may be terminated by receptor endocytosis.

Under normal conditions, endothelial NO synthase (eNOS) is the primary source of NO, the endothelium-dependent vasodilator that is a critical regulator of both vascular tone and VSMC proliferation. Inducible NOS (iNOS) becomes activated under inflammatory conditions (40). We investigated whether NOS expression or phosphorylation were modified by dynasore or TNF-α. iNOS expression was not increased by TNF-α, but this effect was not significant, and dynasore did not modify the response (Fig. 6A). Although neither TNF-α alone nor dynasore significantly affected total eNOS expression, a trend toward this was observed in both cases, and expression was significantly reduced by the combination of TNF-α and dynasore (Fig. 6A). In view of the trend toward a reduction in total eNOS expression, but an associated increase in phosphorylation, we sought to determine whether these effects of TNF-α and dynasore impacted NO bioavailability. We used DAF-2 fluorescence to quantify NO production by cultured endothelial cells and found that, although TNF-α reduced production, this effect was not modified by dynasore (Fig. 6B). Collectively, these data do not support a change in NO metabolism as the primary mechanism for enhancement of TNF-α-induced endothelial dysfunction by dynasore.

TNF-α induces apoptotic and/or necrotic cell death depending on cell type and conditions (14, 16, 33). We therefore explored whether inhibition of endocytosis during TNF-α exposure of endothelial cells was associated with increased apoptosis and cell death. Cleavage of poly(ADP-ribose) polymerase (PARP) was measured as a marker of apoptosis, but it was not significantly altered by the combination of TNF-α and dynasore (Fig. 6C). TUNEL staining of intact mesenteric arteries was also not significantly altered by incubation in TNF-α with or without dynasore, suggesting the lack of a dramatic impact on apoptosis (data not shown). We next assessed cell viability using the SRB assay (59). Incubation for 24 h in dynasore plus TNF-α reduced cell viability more than...
TNF-α incubation alone (Fig. 6D). These data were confirmed by cell counting using trypan blue staining of the endothelial cells (Fig. 6E). The results suggest that endothelial dysfunction induced by TNF-α and dynasore is related to endothelial cell death but not by an apoptotic mechanism.

**NF-κB activation by TNF-α.** We explored whether NF-κB activation by TNF-α, which depends almost completely on endosomal reactive oxygen production in VSMC (37), is affected by inhibition of endocytosis in endothelial cells. Dynasore (15 μM) caused only a modest (~30%) reduction in TNF-α-induced NF-κB activation (Fig. 7A), indicating that in endothelial cells NF-κB activation may be associated with both endocytosis-dependent and -independent pathways. Since TNF-α can cause both NF-κB-dependent proliferation and JNK-dependent cell death, we examined the relationship between NF-κB activation and MAPK activity in cultured endothelial cells. To investigate effects of JNK, p38, and ERK on NF-κB activity, inhibitors of JNK (SP600125), p38 (SB203580), and ERK (U0126) were once again employed. After 6 h incubation, TNF-α enhanced NF-κB activity by ~40-fold, and JNK inhibition more than doubled this activation, whereas the other MAPK inhibitors had no effect (Fig. 7B). Given the ability of dynasore to enhance JNK phosphorylation (Fig. 5) and reduce NF-κB activation (Fig. 7A), the relationship between JNK activity and endocytosis-dependent NF-κB activation was further assessed. We considered whether the effect of dynasore on NF-κB activation might be indirect, mediated by enhanced JNK activation (Fig. 5). Dynasore still decreased NF-κB activation even in the setting of JNK inhibition (Fig. 7C), and the magnitude of this effect was similar to that observed under control conditions (Fig. 7A). This suggests that the inhibitory impact of dynasore on NF-κB activation is not mediated by enhanced JNK activation.

In view of the impressive and apparently selective impact of JNK inhibition on NF-κB signaling, we investigated the time dependence of this effect. NF-κB activation in response to TNF-α was maximal at 6 h and returned essentially to baseline after 48 h. However, JNK inhibition markedly potentiated NF-κB activation in response to TNF-α. This enhancement was fully maintained for 24 h, and after 48 h NF-κB activation was still roughly as high as the peak activation observed in the absence of SP600125 (Fig. 7D).

NF-κB controls transcription of hundreds of genes related to the inflammatory response (9). One of these genes is intercellular adhesion molecule-1 (ICAM-1). We measured ICAM-1 protein expression in cultured endothelial cells to determine whether the increment in NF-κB activation by TNF-α caused by SP600125 translated to significant effect on protein expression. As shown in Fig. 7E, ICAM-1 protein expression after 24...
was significantly enhanced by SP600125 plus TNF-α, compared with TNF-α stimulation alone.

**DISCUSSION**

TNF-α impairs endothelium-dependent relaxation (15, 61, 62), but the role of TNFR endocytosis in endothelial TNF-α signaling has not been previously defined. When dynamin activity was inhibited by dynasore, prolonged TNF-α exposure leads to much more severe endothelial dysfunction. This effect did not appear to be related to an effect on NO production but was associated with enhanced JNK and p38 activation and increased endothelial cell death. Importantly, JNK provided a potent negative influence on NF-κB activation by TNF-α. This novel effect of JNK may represent an important new mechanism by which JNK shifts TNF-α signaling away from proliferation and promotes cell death (Fig. 8).

Receptor endocytosis can terminate signaling by removing an activated receptor from the plasma membrane and targeting the receptor for degradation within lysosomes. However, endocytosis may also be required to facilitate distinct signaling events that localize to endosomes. We have shown previously that, in VSMC, TNFR endocytosis is dynamin dependent (38, 52) and that endosomal ROS production, which is essential to both NF-κB and ERK1/2 activation (8), occurs within this compartment (38). In contrast to what was seen in murine VSMC, in the present study, TNFR endocytosis is only partly associated with TNF-α-mediated NF-κB activation in murine endothelial cells.

MAPKs are activated in response to various stimuli, including cytokines and environmental stresses. Although ERK1/2 generally supports cell survival, migration, and proliferation (53), JNK and p38, which are activated by stressors such as oxidative conditions, ultraviolet radiation, and cytotoxins, promote cell death (36). In the present study, TNF-α caused phosphorylation of JNK, p38, and ERK, as previously reported (1, 39). However, only JNK and p38 activation by TNF-α were enhanced by dynasore. These data suggest that receptor endocytosis may terminate JNK and p38 activation that normally occurs at the plasma membrane. This finding raises the possibility that influences that globally impair or delay endocytosis (31, 44, 54) can promote JNK- and p38-dependent cell death over ERK1/2- and NK-κB-dependent proliferation of endothelial cells.

TNF-α has been reported to have variable effects on eNOS. TNF-α can decrease eNOS protein expression in endothelial cells and intact vessels (2, 62), but in another setting no change in eNOS activity was observed (28). It has been reported that JNK inactivates eNOS (4, 43); however, it is not known specifically whether TNF-α-induced JNK activation inhibits eNOS. Dynasore has been used to inhibit dynamin-dependent endocytosis, and it is a highly selective inhibitor (7, 21). Relatively little is known regarding the impact of dynasore on
Fig. 7. NF-κB activation by TNF-α is reduced by dynasore and enhanced by JNK inhibition in endothelial cells. NF-κB activity was measured as described in METHODS.

A: after 1 h of incubation with DMSO or dynasore (15 or 60 μM), TNF-α was applied for 6 h. B: inhibitors were incubated for 30 min before TNF-α stimulation. After 6 h of incubation with TNF-α, SP600125 potentiated NF-κB activation, but the other inhibitors (U0126, SB203580) did not modify the effect of TNF-α. C: inhibition of NF-κB activation by dynasore persists in the presence of SP600125. *Significant difference vs. control (P < 0.05). †Significant difference (n = 4; P < 0.05).

D: time dependence of enhancement of NF-κB activation in response to TNF-α by SP600125. *Significant difference vs. TNF-α in each time period (n = 4–6; P < 0.05).

E: after 24 h of incubation with DMSO or SP600125 and TNF-α, endothelial cell lysates were Western blotted for ICAM-1 and tubulin proteins. Bar graph shows the relative abundance of ICAM-1 normalized to tubulin (n = 4). *P < 0.05, significant difference vs. control (DMSO only); **P < 0.05, significant difference.
ROS is not established. Further investigation is required to determine whether the TNF-α-induced endothelial cell death that is potentiated by endocytosis inhibition is ROS dependent.

NF-κB inhibition downregulates JNK (13, 55), and NF-κB activation protects against cell death induced by TNF-α (14, 55). However, an inhibitory effect of JNK on TNF-α-induced NF-κB activation has not previously been reported. We found that JNK inhibition dramatically increased NF-κB activation by TNF-α in endothelial cells. This effect was striking in that it impacted both the magnitude (doubled) and duration of NF-κB activation (Fig. 7B). After 48 h of sustained exposure to TNF-α, NF-κB activity had returned approximately to baseline under control conditions. In contrast, in the presence of SP600125, activity after 48 h remained almost as high as it was at the peak of the response to TNF-α only (observed after 6 h of exposure). Thus the ability of JNK activation to cause cell death may be related to a combination of direct activation of pro-death signaling, combined with potent impairment of pro-proliferative signaling. The relative importance of these two influences remains to be determined. The mechanism by which JNK regulates TNF-α-induced NF-κB activation and inflammatory signaling.

In the vasculature, endothelial cells control the tone of the underlying VSMC by releasing various relaxing and contracting factors (17) and play an important role in the balance and regulation of regional blood flow (50). Impaired endothelial function activates the immune response and contributes to arterial inflammation. A fine balance of proliferation, differentiation, and apoptosis in the vascular wall controls the development and progression of vascular lesions. TNF-α is present in increased concentrations at sites of vascular injury and participates in lesion formation through signal transduction regulating both endothelial cells and VSMC (25, 46). TNF-α induces apoptosis in human aortic endothelial cells, whereas it promotes proliferation in VSMC (47). Similarly, the present data demonstrate that TNF-α causes cell death in mouse mesenteric endothelial cells, whereas previous work demonstrated proliferation to be the dominant response of murine aortic VSMC (8). NF-κB activation is partly endocytosis dependent in endothelial cells, but this requirement may not be as strict as it is in VSMC (35, 37, 38). Thus it is important to further explore the complex impact of endocytosis on TNF-α signaling, since it is clearly cell-type dependent. Carefully defining the subcellular localization of TNFR signaling in endothelial cells vs. VSMC may reveal novel strategies for prevention of vascular inflammation.

**Perspectives**

TNF-α has acute deleterious effects on cardiovascular function in the setting of sepsis and also plays a critical role in the chronic development of coronary artery disease and aneurysm formation. Antibodies that disrupt TNF-α signaling (Eliquence, others) constitute first-line therapy for arthritis, psoriasis, and Crohn’s disease. Unfortunately, they disrupt all aspects of TNF-α signaling and increase the risk of infection. The next generation of anti-TNF-α agents will need to target specific aspects of TNF-α signaling. The present study demonstrates the impact of endocytosis on the balance between TNF-α-
induced, MAPK-dependent cell death and NF-κB-dependent cell survival. The role of endocytosis in these pathways has not been previously defined in the intact blood vessel or in cultured cells. Identification of spatial and temporal differences between pro-survival and pro-death signals may provide new targets for fine tuning of anti-inflammatory therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.C. and H.N.N. performed experiments; H.C. analyzed data; H.C. interpreted results of experiments; H.C. and H.N.N. prepared figures; H.C. drafted manuscript; H.C. and F.S.L. edited and revised manuscript; F.S.L. approved final version of manuscript.

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