Activation of Tumor Necrosis Factor-α-converting Enzyme-mediated Ectodomain Shedding by Nitric Oxide*

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Ectodomain shedding of cell surface proteins is an important process in a wide variety of physiological and developmental events. Recently, tumor necrosis factor-α-converting enzyme (TACE) has been found to play an essential role in the shedding of several critical surface proteins, which is evidenced by multiple developmental defects exhibited by TACE knockout mice. However, little is known about the physiological activation of TACE. Here, we show that nitric oxide (NO) activates TACE-mediated ectodomain shedding. Using an in vitro model of TACE activation, we show that NO activates TACE by nitrosation of the inhibitory motif of the TACE prodomain. Thus, NO production activates the release of cytokines, cytokine receptors, and adhesion molecules, and NO may be involved in other ectodomain shedding processes.

Ectodomain shedding is an essential phenomenon involved in the cleavage and release of cell membrane-bound molecules ranging from Alzheimer’s amyloid precursor protein to angiotensin-converting enzyme (1). Shedding of ligand/receptor families is involved in diverse processes such as inflammation, hematopoiesis, and normal development (2, 3). Ectodomain shedding can be stimulated by protein kinase C activation and endotoxin (4, 5). TACE1 is a member of a disintegrin and metalloproteinase (ADAM) family, a group of unique zinc-binding transmembrane metalloproteinases (6–8). TACE has been shown to mediate cleavage of TNFα as well as a variety of ectodomains including the TNF p75 receptor, L-selectin, and nitrosation of the inhibitory motif of the TACE prodomain. TACE activity is regulated by the cysteine in the cleavage and release of cell membrane-bound molecules ranging from Alzheimer’s amyloid precursor protein to angiotensin-converting enzyme (1). Shedding of ligand/receptor families is involved in diverse processes such as inflammation, hematopoiesis, and normal development (2, 3). Ectodomain shedding can be stimulated by protein kinase C activation and endotoxin (4, 5). TACE1 is a member of a disintegrin and metalloproteinase (ADAM) family, a group of unique zinc-binding transmembrane metalloproteinases (6–8).

TACE contains a consensus cysteine switch motif in the prodomain, and it has been shown previously that the cysteine in this portion of the molecule is required for the inhibition of TACE activity (18). In the present study, we tested the hypothesis that NO, a molecule produced in a variety of inflammatory conditions, regulates TACE activity and ectodomain shedding.

Experimental Procedures

Chemicals—(Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)-amino]-diazen-1-ium-1,2-dilolate (PAPA/NO), N6-monomethyl-l-arginine (NMA), and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one were obtained from Alexis Biochemicals (San Diego, CA). Actinomycin D and oxyhemoglobin were from Sigma. Murine interferon-γ was provided by Genentech (South San Francisco, CA). Escherichia coli LPS 026:B6 was from Difco. All other chemicals were purchased from Sigma.

Cell Culture—Mono Mac 6 and Jurkat cells were cultured in RPMI 1640 with 15 and 5% fetal bovine serum (Life Technologies, Inc.), respectively. IEC-6 cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. Mouse TACE Azn/Δzn or control TACE +/+ monocytes were maintained in RPMI 1640 with 10% fetal bovine serum, 20 ng/ml granulocyte-macrophage colony-stimulating factor, and 100 μM β-mercaptoethanol. Lung macrophages were obtained from 129 × C57BL/6 mice or iNOS−/− mice (19) by lung lavage with warmed phosphate-buffered saline plus 0.5 mM EDTA. Cells were >95% viable macrophages (determined by trypan blue staining) and were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. All media contained 1% penicillin/streptomycin, and cells were maintained at 37°C and 5% CO2. TNFα production was assayed in Mono Mac 6 cells by stimulation with a combination of 20 ng/ml LPS and 60 ng/ml PMA for 3 h in the presence of varying concentrations of PAPA/NO. Experiments were also performed with and without 100 μM TAPI (Peptides International, Louisville, KY), a hydroxamate-based metalloproteinase inhibitor that tightly binds to metalloproteinase catalytic domain, or 5 μM oxyhemoglobin. To assess shedding of TNF p75 receptors (Mono Mac 6 cells) or L-selectin (Jurkat cells) by NO, cells were incubated with varying concentrations of PAPA/NO with and without 100 μM TAPI. Certain experiments were performed in the presence of 2 μg/ml actinomycin D to rule out a transcriptional effect of PAPA/NO on ectodomain shedding.

Co-culture of Mono Mac 6 and IEC-6 Cells—IEC-6 cells, a rat intestinal epithelial cell line, were stimulated with both murine interferon-γ (10 units/ml) and LPS (20 ng/ml) for 18 h to induce NO production. NO production by this cell line was monitored by the Griess reaction assay. Then, Mono Mac 6 cells were co-cultured with this cell line in the presence of continuous murine interferon-γ and LPS stimulation for 3 h. Human TNF p75 receptors in the culture medium and Mono Mac 6 cell lysates were measured by ELISA. Experiments were also performed in the presence of 100 μM NMA or 5 μM HbO2 to antagonize NO production or activity, respectively.

ELISA—Cell lysates were prepared in a lysis buffer containing phos-
phosphate-buffered saline, pH 7.2, 1% Triton X-100, and a proteinase inhibitor mixture (Roche Molecular Biochemicals). Cell lysates and supernatants were harvested at 2 h (TNF p75 receptors and L-selectin) or 3 h (TNFα). An ELISA was used to measure shed and cell-associated human TNFα, L-selectin (R & D Systems, Minneapolis, MN), human TNF p75 receptors (BIOSOURCE, Camarillo, CA), and mouse TNF p75 receptors (Genzyme, Cambridge, MA). Experiments with recombinant murine TNF p75 receptors and supernatants of PMA-stimulated mouse monocytes confirmed the specificity of the human TNF p75 receptor ELISA.

Flow Cytometry—Surface TACE was assessed by staining Mono Mac 6 cells with M222 (8), a monoclonal antibody against human TACE, or with isotype control followed by incubation with a secondary phycoerythrin-conjugated rat anti-mouse antibody (PharMingen, San Diego, CA). Surface TNF p75 receptors were detected on TACE +/+ and TACE ΔZn/ΔZn by staining with a biotinylated hamster anti-mouse TNF p75 receptor (Genzyme) or with isotype control followed by incubation with streptavidin-phycocerythrin (PharMingen). Cells were analyzed on a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

Recombinant TACE Cleavage Assay and Determination of Nitrosothiol—Purified TACE was a gift of Dr. Roy Black (Immunex Corp., Seattle, WA). TACE activity was determined by its ability to cleave a 12-amino acid peptide containing a known TACE cleavage site, SPLAQ*VHLPQP, with a diisopropylfluorophosphatase motif at the N terminus to enhance absorbance at 350 nm. The inhibitory peptide (TIP) was synthesized from the prodomain sequence PKVCAYLK (20). All reactions were initiated in 10 mM Tris buffer (pH 7.4) that had been sparged with argon. To inhibit TACE, TIP (1–500 μM) for 1 h at room temperature in 50 μl of buffer. To test the ability of the nitric oxide donor to reverse TACE inhibition, PAPA/NO (Alexis Biochemicals) was added to the solution for a final concentration of 0.5 or 1 μM (1 h, room temperature). Finally, 50 μl of a TACE cleavage peptide solution was added for a final concentration of 200 nm (15 min, 37 °C). The reaction was stopped by adding 10 μl of 2 N HCl.

RESULTS

Enhancement of TNFα Sheding by NO—To test the possible involvement of NO in activation of TACE, we added an exogenous source of NO, PAPA/NO, to LPS/PMA-stimulated Mono Mac 6 cells, a human monocyte cell line that expresses TACE (8). This treatment resulted in a dose-dependent increase in secreted TNFα in the culture supernatant. This effect is due to the NO donor because these cells do not produce NO, and the enhanced secretion of TNFα by PAPA/NO is blocked by 5 μM oxyhemoglobin, a scavenger of free NO (Fig. 1a). This enhanced secretion of TNFα is associated with a decrease in cell-associated TNFα 2 h after stimulation with LPS and PMA (Fig. 1b). Thus, at any given level of cell-associated TNFα over time, the level of secreted TNFα is higher in cells exposed to PAPA/NO (Fig. 1c). Thus, NO enhances TNFα processing across the cell membrane. We did not observe significant differences in cell-associated TNFα at 3 h; however, this may be because NO stabilized TNF mRNA (as reported by Wang et al. (12)), resulting in an increase in cell-associated TNFα at this time point. TAPI, a metalloproteinase/TACE inhibitor, blocks both LPS/PMA-induced and NO-induced increases in TNFα processing (data not shown). These data suggest that NO can enhance TNF processing by enhancing TACE activity.

Effects of NO on Other Ectodomain Sheding—Unlike TNFα, which requires transcriptional stimulation by LPS and PMA (a known activator of TACE activity) in Mono Mac 6 cells, we investigated whether NO can activate shedding of constitutive TACE-dependent ectodomains. Specifically, we quantitated the shedding of TNF p75 receptor from Mono Mac 6 cells and of L-selectin from Jurkat T cells. Both of these ectodomains are cleaved by TACE (3). Therefore, releases of preformed TNF p75 receptor and L-selectin were used as sensitive markers for TACE cell surface activity (3) and do not require transcriptional activation or confounding complications associated with PMA stimulation. When Mono Mac 6 cells were exposed to PAPA/NO, we observed a dose-dependent shedding of the TNF p75 receptor from the cell surface over a 2-h time period (Fig. 2a). These concentrations of PAPA/NO, which result in steady-state NO concentrations in the micromolar range (22), did not cause cytotoxicity as defined by lactate dehydrogenase release (data not shown). Moreover, soluble TNF p75 receptor shedding from Mono Mac 6 cells was completely blocked by TAPI (Fig. 2a), and shedding was not observed with degraded PAPA/NO. To rule out the possibility that PAPA/NO enhanced TNF p75 receptor transcription and production, cell-associated TNF p75 receptors were measured in the cell lysates, which showed that PAPA/NO treatment caused a dose-dependent decrease of cell-associated TNF p75 receptor (Fig. 2b). Incubation of Mono Mac 6 cells with PAPA/NO in the presence of 2 μg/ml actino-
mycin D did not alter NO-induced shedding of the TNF p75 receptor. NO-induced shedding was blocked by HbO₂ (Fig. 2c). Such a reciprocal change of shed versus cell-associated TNF p75 receptors in PAPA/NO-treated cells demonstrates that NO caused TNF p75 receptor cleavage from the cell membrane independent of transcriptional activity.

In further support of NO activation of TACE-mediated ectodomain shedding, we observed PAPA/NO-mediated release of L-selectin from the surface of Jurkat T-cells (Fig. 2d). As was the case for TNF p75 receptor shedding in Mono Mac 6 cells, no increase in shedding was observed with the parental compound, degraded PAPA/NO, or in the presence of TAPI (data not shown). NO-induced shedding was also seen after treatment of the cells with a nitrosothiol NO donor, S-nitroso-N-acetylpenicillamine, at a 20-fold lower concentration (5 μM). This effect was not blocked by HbO₂, which demonstrates that NO-induced TACE-mediated ectodomain shedding can also occur via transnitrosation (23).

Enhancement of Ectodomain Shedding by NO in Pathophysiological Settings—To investigate whether endogenous NO can activate TACE-mediated ectodomain shedding, we induced TNF p75 receptor shedding in lung macrophages from control mice or inducible nitric oxide synthase knockout (iNOS −/−) mice (19). Upon stimulation with 20 ng/ml LPS, the control cells produced between 10 ± 50 μM NO (data not shown). LPS-induced NO production was associated with a significant increase in TNF p75 receptor shedding (Fig. 3a). However, iNOS −/− macrophages failed to make detectable NO, and they did not shed TNF p75 receptors (Fig. 3a) despite the presence of TACE and TNF p75 receptors on the cell surface as measured by flow cytometry (Fig. 3b).

To investigate whether NO can act in a paracrine fashion to increase TACE activity in human macrophages, which do not produce NO in culture, we co-cultured Mono Mac 6 cells with a rat intestinal epithelial cell line, IEC-6 cells, which produce NO under stimulation with murine interferon-γ and LPS (24, 25). After 18 h, NO production by IEC-6 cells was confirmed by Griess reactivity after reduction of nitrate to nitrite. Stimulated cells produced 10.68 ± 1.28 μM nitrate plus nitrite compared with 2.52 ± 0.42 μM in unstimulated cells and 2.98 ± 1.28 μM in stimulated cells in the presence of 1 mM NMA (a competitive inhibitor of NO synthase). A 5-h co-culture of Mono Mac 6 cells with stimulated IEC-6 cells resulted in significant human TNF p75 receptor shedding from Mono Mac 6 cells compared with co-culture with unstimulated IEC-6 cells (Fig. 3c). Direct treatment of Mono Mac 6 cells with murine interferon-γ and LPS did not alter human TNF p75 receptor shedding (112 ± 9.8 pg/ml/5 h versus 120 ± 13.6 pg/ml/5 h in control cells). HbO₂ (5 μM) inhibited activation of TNFα p75 receptor shedding (Fig. 3c), confirming the direct role of NO in TACE activation.

Involvement of TACE in NO-induced Ectodomain Shedding—Although TACE mediates TNF p75 receptor and L-selectin shedding (3), other sheddases could contribute to TNF p75 receptor shedding under these conditions. To further define that the ectodomain shedding facilitated by NO is mediated by TACE specifically, we utilized monocytes derived from...
mice with a targeted mutation (TACE ΔZn/ΔZn) encoding an in-frame deletion of the zinc binding domain in the TACE gene (3). This domain is essential for TACE activity, and TACE ΔZn/ΔZn cells have been shown to be deficient in the processing of TACE cleavage substrates (3). In the control monocytic cell line (TACE +/+), PAPA/NO resulted in dose-dependent shedding of Tfn p75 receptors in murine TACE +/+ cells, whereas no such shedding was observed in TACE ΔZn/ΔZn cells (n = 4; ND, none detected; *, p < 0.05 compared with the base-line value). Shedding of Tfn p75 receptors from TACE +/+ monocytes was blocked by HbO 2 or DAN (n = 4; *, p < 0.05 compared with the other groups). Neither DAN nor HbO 2 itself affected base-line DTACE bTNF p75 receptor shedding in these cells (Fig. 4 2,3-diaminonaphthalene (DAN) blocked PAPA/NO-induced nitrosation of NO in the presence of oxygen forms the nitrosating compound, N2O3, which reacts with and is neutralized by DAN (26). NO-induced Tfn p75 receptor shedding was not observed in TACE ΔZn/ΔZn monocytes (Fig. 4a). NO-induced Tfn p75 receptor shedding in the wild type TACE +/+ cells was corroborated by analyzing TACE ΔZn/ΔZn and TACE +/+ cells by flow cytometry for surface Tfn p75 receptor expression. PAPA/NO (1 mM) resulted in a significant decrease in the mean channel fluorescence for surface Tfn p75 receptor in TACE +/+ cells (Fig. 4c, left panel) but caused no change in surface Tfn p75 receptor expression in TACE ΔZn/ΔZn cells (Fig. 4c, right panel).

Direct Activation of TACE by NO—NO is well known as a signal transduction molecule, exerting many of its biological activities. However, NO-induced ectodomain shedding did not appear to require any of the known potential signaling pathways for NO. NO-induced Tfn p75 receptor shedding was not affected by an inhibitor of cyclic GMP (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), 8-bromo cyclic GMP, chelerythrin (a calcium-dependent protein kinase C inhibitor), genistein (a tyrosine kinase inhibitor), or dibutyl cyclic AMP, which increases intracellular cAMP (data not shown). Moreover, flow cytometry analysis with a monoclonal antibody against the catalytic domain of TACE revealed that PAPA/NO does not alter the TACE signal on the cell surface (mean channel fluorescence, 53 ± 4.3 versus 55 ± 5.2 after a 2-h exposure to 1 mM PAPA/NO). These data suggest that NO increases ectodomain shedding by direct activation of latent TACE.

Further experiments on the activation of TACE by NO were performed using a recombinant processed TACE that lacks the prodomain and is constitutively active (7). Incubation of this protein with NO resulted in no further enhancement of TACE activity using a peptide cleavage assay (7) (data not shown). However, these data do not exclude the possibility that NO could nitrosate the thiol in the prodomain covering the catalytically active site and thereby activate latent TACE. To test this possibility, we constructed an in vitro model of unprocessed TACE using recombinant active TACE in combination with a TIP. This peptide, PKVCYGLK, contains the consensus cysteine switch motif of the TACE prodomain that inhibits TACE activity, as described previously (7, 8, 20). A TACE cleavage peptide (TCP), which contains the TfnA TACE cleavage site (7, 8) 2,4-dinitrophenyl-SPLAQAVHLPQP, was used as the substrate for TACE cleavage activity. Prior N-terminal sequencing has confirmed that TACE cleaves this peptide at the alanine/valine site in the TCP (7). The uncleaved TCP migrated as a single peak that eluted at approximately 18 min by HPLC (Fig. 5). Incubation of TCP with TACE (15 min, 37 °C) resulted in almost complete cleavage of the TCP (95 ± 1.2%) with a distinct cleavage product that eluted at approximately 17 min. Preincubation of TACE with TIP significantly decreased TACE activity to 20 ± 2.6% cleavage of the TCP (Fig. 5, n = 5). Incubating the TACE/TIP complex with 1 mM PAPA/NO reversed the inhibitory effect of TIP, restoring the cleavage of TCP to 91 ± 3.1% (Fig. 5). Incubation of TIP alone with PAPA/NO prior to incubation with TACE also abolished the inhibitory effect of TIP (data not shown). The inhibitory action of TIP exposed to PAPA/NO was reconstituted by subsequent addition of 1 mM diithiothreitol (data not shown). These data are consistent with nitrosation of the cysteine at the cytoplasmic side of the inhibitory motif of the prodomain, which thereby prevents the thiol group from coordinating with the Zn2+ atom in the catalytic site of TACE.

To confirm nitrosation of the cysteine of the prodomain of TACE as a regulatory mechanism of TACE activation, we analyzed nitrosothiol formation of TIP using HPLC with an absorbance at 335 nm (21). Incubation of TIP with NaNO2 in the presence of 0.12 M HCl (positive control for nitrosothiol formation) or with 1 mM PAPA/NO (1 h, room temperature) resulted in a nitrosotetrahydrothiophenol product that eluted at 10 min (Fig. 6). Untreated TIP or PAPA/NO alone resulted in no absorbance peak at 335 nm (Fig. 6). Consistent with a nitrosated product, the absorbance peak of TIP incubated with PAPA/NO was extinguished by 10 mM HgCl2, which removes NO from the nitrosated thiol (21). These results indicate that NO can directly nitrosate TACE prodomain, leading to activation of latent TACE in vitro.
DISCUSSION

Most matrix metalloproteinases and ADAMs are synthesized in a latent form (27, 28). This latency is the result of an intramolecular complex between the cysteine residue in the zinc switch domain and the zinc atom in the catalytic domain. The amino acid sequences surrounding the critical cysteine residue and the region containing two of the putative histidine zinc-binding ligands are highly conserved (27). Several reactive free radicals (including NO and other reactive nitrogen oxides) have been suggested to activate metalloproteinases via attack of the cysteine thiol group in the zinc switch thereby exposing the active zinc site (26, 29–31). Although this mechanism is operative in many matrix metalloproteinases, this regulation has not been shown for activation of members of the ADAM family of metalloproteinases. Alkylation of this prodomain thiol by 4-aminophenylmercuric acetate can activate full-length recombinant TACE in solution (18). However, whether the modification of the cysteine residue in the inhibitory prodomain plays a physiological role in regulation of TACE activity has not been shown.

In these studies, we demonstrate that NO provided by a NO donor or generated in a physiological setting (either endogenously or exogenously produced) is a critical factor in TACE-mediated ectodomain shedding. This is the first time that NO has been shown to activate ectodomain shedding and play a role in the physiological regulation of TACE activity. Moreover, we have shown that NO can form a nitrosothiol with the cysteine residue in the inhibitory prodomain of TACE, which results in an enzymatically active form of TACE in vitro. In addition to the cysteine switch complex, TACE contains another cysteine-rich domain near its transmembrane segment (7). Chemical modification of this cysteine-rich domain has been shown to affect TACE enzymatic activity (18). Thus, nitrosation of thiol groups in this domain could also contribute to the enhancement of TACE-mediated ectodomain shedding by NO. In the present study, we found that direct incubation of recombinant TACE with NO did not alter TACE-mediated cleavage. However, NO restored TACE enzymatic activity inhibited by a peptide mimicking TACE prodomain, suggesting that NO activates TACE via alteration of the cysteine switch. Cell surface TACE is reported to be active in the unstimulated condition (7), and active TACE presumably maintains a basal level of ectodomain shedding, which is required for normal cell growth and metabolism. Nevertheless, LPS or protein kinase C activation can further enhance TACE-mediated ectodomain shedding (3, 7, 8). Upon situations such as inflammation, it is feasible that NO and other factors up-regulate sheddases to regulate cell signaling and adhesion. In fact, it has been previously demonstrated that NO is a critical regulator of cell adhesion to the endothelium (32, 33). In this study, we did not observe an increase in cell surface TACE by NO at the level of

![Fig. 5. PAPA/NO directly restores TACE enzymatic activity inhibited by TIP.](image)

**FIG. 5.** PAPA/NO directly restores TACE enzymatic activity inhibited by TIP. Nitric oxide activates latent TACE. The TCP elution peak was almost completely cleaved by TACE (TCP + TACE). Preincubation of TACE with TIP inhibited TCP cleavage (TACE + TIP + TCP). PAPA/NO reversed the TACE inhibition by TIP (TACE + TIP + TCP + PAPA/NO).

![TACE prodomain nitrosation assay](image)

**FIG. 6.** Nitrosation of TIP by PAPA/NO. TIP was nitrosated by nitrite under acidic conditions (TIP + Nitrite + HCl). The nitrosothiol peak was also observed in PAPA/NO-treated TIP (PAPA/NO + TIP). No nitrosothiols were detectable in TIP or PAPA/NO alone. Note that the addition of HgCl₂ reversed nitrosation of cysteine in PAPA/NO-treated TIP (PAPA/NO + TIP + HgCl₂).
flow cytometry. Since NO is a cell membrane-diffusible molecule, we postulate that NO activates an intracellular pool of preformed latent TACE. In addition, the large diffusion distance of NO in tissue suggests activation of ectodomain shedding in multiple cells by a single cell producing NO (34). In summary, these findings suggest a novel role for NO in modulating TACE-dependent cytokine secretion, cytokine responsiveness, and ectodomain shedding and partially explain the augmented adhesion of leukocytes in the setting of reduced levels of NO (32).

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