Reactive Oxygen Species and p38 Mitogen-activated Protein Kinase Mediate Tumor Necrosis Factor α-Converting Enzyme (TACE/ADAM-17) Activation in Primary Human Monocytes*

Background: The mechanisms responsible for up-regulation of TNFα-converting enzyme (TACE) catalytic activity in primary human monocytes have not been elucidated.

Results: TACE activation by lipopolysaccharide was dependent on reactive oxygen species (ROS) and the p38 MAPK pathway.

Conclusion: ROS mediate TACE catalytic activation indirectly through the p38 pathway.

Significance: This redefines the mechanisms of TACE activation in primary cells with a physiological stimulus.

Tumor necrosis factor α-converting enzyme (TACE) is responsible for the shedding of cell surface TNF. Studies suggest that reactive oxygen species (ROS) mediate up-regulation of TACE activity by direct oxidation or modification of the protein. However, these investigations have been largely based upon nonphysiological stimulation of promonocytic cell lines which may respond and process TACE differently from primary cells. Furthermore, investigators have relied upon TACE substrate shedding as a surrogate for activity quantification. We addressed these concerns, employing a direct, cell-based fluorometric assay to investigate the regulation of TACE catalytic activity on freshly isolated primary human monocytes during LPS stimulation. We hypothesized that ROS mediate up-regulation of TACE activity indirectly, by activation of intracellular signaling pathways. LPS up-regulated TACE activity rapidly (within 30 min) without changing cell surface TACE expression. Scavenging of ROS or inhibiting their production by flavoprotein oxidoreductases significantly attenuated LPS-induced TACE activity up-regulation. Exogenous ROS (H2O2) also up-regulated TACE activity with similar kinetics and magnitude as LPS. H2O2 and LPS-induced TACE activity up-regulation were effectively abolished by a variety of selective p38 MAPK inhibitors. Activation of p38 was redox-sensitive as H2O2 caused p38 phosphorylation, and ROS scavenging significantly reduced LPS-induced phospho-p38 expression. Inhibition of the p38 substrate, MAPK-activated protein kinase 2, completely attenuated TACE activity up-regulation, whereas inhibition of ERK had little effect. Lastly, inhibition of cell surface oxidoreductases prevented TACE activity up-regulation distal to p38 activation. In conclusion, our data indicate that in primary human monocytes, ROS mediate LPS-induced up-regulation of TACE activity indirectly through activation of the p38 signaling pathway.

Ectodomain shedding describes the proteolytic cleavage of transmembrane proteins to their soluble forms (1). Tumor necrosis factor alpha-converting enzyme (TACE) was the first mammalian sheddase to be discovered and is a member of the ADAM family of metalloproteases (a disintegrin and metalloprotease; ADAM-17) (2, 3). TACE is the primary physiological sheddase responsible for cleaving membrane TNF to its soluble form (4, 5). TACE has also been shown to be involved in the cleavage of other immunologically relevant substrates, including both TNF receptors and L-selectin (6, 7). Consequently, TACE is in a position to regulate the pleiotropic biology of TNF, a cytokine known to play a major role in both acute (8) and chronic (9) inflammation. An understanding of the regulation of TACE activity therefore provides insight into the pathophysiology of inflammatory disease and may aid the development of TACE-directed therapeutics. However, previous investigations into the regulation of TACE activity have largely been limited to the nonphysiological stimulation of cell lines and have yielded conflicting conclusions.

TACE is initially synthesized as a zymogen, with an inhibitory prodomain maintaining the active site zinc ion in a catalytically inactive state (10). Prodomain removal is essential for active TACE function (11). Some cell lines may express immature, prodomain-containing TACE at the cell surface (12, 13), prompting investigators to suggest that prodomain removal/
modification may be a mechanism for regulating TACE activity (14, 15). In contrast, in primary human cells, the prodomain is removed intracellularly (12, 16, 17), and all cell surface TACE is expressed in the mature form (2), rendering prodomain modification an unlikely mechanism for TACE activity up-regulation in physiological states.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the up-regulation of TACE shedding activity in a variety of experimental systems using cell lines (14, 15, 18, 19). Zhang et al. suggested that in an immature monocyte cell line, PMA-induced ROS/RNS attack the cysteinyl thiol of the prodomain, nullifying its inhibitory effect on TACE (14, 15). However, the role of ROS in stimulation-induced up-regulation of TACE activity on primary cells is unknown. Furthermore, if ROS are involved, their mechanism of action should not involve a “direct” oxidizing effect on the prodomain because it was already removed from the cell surface TACE. ROS have emerged as essential effectors of a variety of intracellular signaling pathways such as the mitogen-activated protein kinase (MAPK) cascades (20, 21). Inhibition of p38 MAPK has been shown to attenuate the shedding of some TACE substrates (22–26). Therefore, we considered that ROS may mediate TACE activity up-regulation “indirectly,” by activating intracellular signaling pathways.

Previous studies of TACE activity regulation have relied upon shedding of TACE substrates as a surrogate for catalytic activity estimation. We previously developed a highly sensitive fluorometric assay to directly quantify cell surface TACE activity (27). We employed this assay to investigate the regulation of TACE activity on primary human monocytes in response to LPS stimulation. We took a pharmacological approach and used freshly isolated monocytes to preserve, as far as possible, their in vivo phenotype. We identified the crucial role of ROS in mediating LPS-induced TACE activity up-regulation in human monocytes. Further investigation revealed that ROS mediate TACE activity up-regulation indirectly, via the activation of p38 MAPK and downstream MAPK-activated protein kinase 2 (MK2). Finally, we explored the ultimate mechanism responsible for TACE activity regulation and implicated disulfide exchange facilitated by cell surface oxidoreductases.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—PBS, dihydrorhodamine 123 (DHR), FCS, and Hanks’ balanced salt solution were purchased from Invitrogen. Sigma supplied the following: saponin, dimethyl sulfoxide, BSA, desferrioxamine (DFO), diphenyleiodonium chloride (DPI), EDTA, H$_2$O$_2$, manganese(III) dimethyl sulfoxide, BSA, desferrioxamine (DFO), diphenyleiodonium chloride (DPI), EDTA, H$_2$O$_2$, manganese(III) dimethyl sulfoxide, BSA, and 2 mM EDTA. RPMI, L-glutamine, FCS, and Hanks’ balanced salt solution were purchased from Invitrogen. Sigma supplied the following: saponin, L-Arginine Methyl Ester hydrochloride (L-NAME) and MK2 Inhibitor III were from Merck. RPMI (Escherichia coli, strain 0111:B4) was from InvivoGen (Wiltshire, UK). EO 1428 was purchased from Tocris Bioscience (Bristol, UK). Biomol International LP (Exeter, UK) supplied the fluorescein-tetramethylrhodamine (FAM-TAMRA) fluorescence resonance energy transfer (FRET) peptide that consists of a TACE-sensitive TNF sequence (FAM-SPLAQAVRSSRK-TAMRA) (27).

R&D Systems supplied fluorophore-conjugated anti-human TACE ectodomain mAb and relevant isotype control; BD Biosciences supplied anti-human CD14 mAb and 7-amino actinomycin D. Fluorophore-conjugated anti-human phospho-p38, phospho-ERK, and phospho-MK2 mAbs were from Cell Signaling Technology.

**Cell Harvest and Purification**—Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor blood by density gradient centrifugation through Ficoll (Axis-Shield PoC AS, Oslo, Norway) in Leukosep tubes (Greiner Bio One Ltd., Gloucester, UK) according to the manufacturer’s instructions. PBMCs were washed and resuspended in separation buffer (Hanks’ balanced salt solution supplemented with 0.1% BSA and 2 mM EDTA). CD14$^+$ monocytes were purified from PBMCs by negative immunomagnetic bead separation according to the manufacturer’s (Miltenyi Biotech, Surrey, UK) instructions. Briefly, nonmonocytes were labeled with a mixture of biotinylated antibodies (against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a) followed by addition of anti-biotin microbeads and anti-CD61 microbeads (to ensure platelet removal) before depletion on a magnetic separation column. Monocytes separated by this method were typically >90% pure CD14$^+$, low side-scatter cells as assessed by flow cytometry.

In acknowledgment of the physiological heterogeneity between individuals, blood was taken from a pool of >10 donors. In addition, the repeats of each experiment were performed with monocytes isolated from a minimum of 3 donors.

**Cell Stimulation**—Monocytes were stimulated at 37°C in Hanks’ balanced salt solution supplemented with 5% FCS at a concentration of 5 × 10$^6$ cells/ml. The kinetics and dose dependence of LPS stimulation were assessed with incubation times varying between 15 min and 3 h and increasing LPS concentrations from 100 pg/ml to 10 μg/ml. To investigate the role of ROS, monocytes were preincubated with NAC (neutral pH adjusted), MnPyP, DPI, DFO, or L-NAME for 30 min before 30-min LPS stimulation. In addition, cells were stimulated with H$_2$O$_2$. MAPK pathways were investigated by pretreating monocytes with p38 MAPK inhibitors (SB203580, SB202190, and EO 1428), MK2 Inhibitor III, or the ERK pathway inhibitor U0126 before stimulation. To inhibit cell surface oxidoreductase, monocytes were pretreated with 4-((N-((S)-glutathionylacetyl)amino)phenylarsenoxide (GSAO), a membrane-impermeable, specific, vicinal thiol blocker (28), for 30 min. Concentrations of all pharmacological agents used (described under “Results”) are considered to be “saturating doses,” consistent with those found within the relevant literature. Dimethyl sulfoxide was used as the vehicle for some agents up to a maximum concentration of 0.4% which had no effect in the relevant experiments (data not shown).

**Measurement of Cell Surface TACE, Intracellular Phospho-p38, and Cell Viability**—Following stimulation, cells were washed, and a small fraction was used for flow cytometric analysis. To quantify TACE expression, cells were stained with anti-human TACE mAb or the relevant isotype control and anti-human CD14 for 10 min at room temperature before being
washed and resuspended in FACS wash buffer (FWB-PBS supplemented with 2% FCS, 5 mM EDTA, and 0.1% sodium azide). CALTAG Counting Beads (Invitrogen) were then added to each sample. For quantification of intracellular phospho-p38, monocytes were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) solution according to the manufacturer’s instructions. Cells were then washed twice in permeabilization/wash buffer (PBS supplemented with 2% FCS, 0.1% sodium azide, and 0.2% saponin) and stained with anti-human phospho-p38 mAb and anti-human CD14 for 30 min at room temperature in the dark, before a further wash and resuspension in permeabilization/wash buffer. Analysis of TACE and phospho-p38 expression was performed with a CyAn ADP Analyzer flow cytometer (Beckman Coulter) and Flowjo software (Tree Star, Ashland, OR). Expression is reported as the mean fluorescence intensity (MFI) of CD14+ low side-scatter gated cells with the appropriate isotype control subtracted. Measurement of phospho-MK2 and phospho-ERK expression was performed as for phospho-p38. Cell viability following stimulation (>90% for all experiments) was assessed by adding the nuclear dye 7-amino actinomycin D to samples prior to acquisition according to the manufacturer’s instructions.

TACE Activity Assay—Following stimulation, monocytes were washed and resuspended in assay buffer (Hanks’ balanced salt solution supplemented with 1% BSA). Monocyte number and viability were assessed as described above before being plated at 1 × 10⁵ cells/well in black 96-well plates (Corning). Cells were plated in duplicate or, number permitting, triplicate. Well volumes totaled 100 μl with 5 μM FAM-TAMRA TNF peptide and an appropriate volume of assay buffer. TACE enzymatic activity was quantified by continuous measurement of fluorescence intensity in a microplate fluorometer (Flx-800; Bio-tek Instruments Inc., Bedfordshire, UK) at 37 °C and at λex 485 nm and λem 535 nm in accordance with the protocol described previously (27). Data were acquired with KC4 data analysis software (Bio-tek Instruments Inc.). Following an initial equilibration period of ~5 min, enzymatic activity was calculated as the gradient of the fluorescence-time plot over a 10-min period, giving values in fluorescence units/min. When applicable, activity was calculated as the mean of multiple wells.

Detection of Intracellular ROS—To investigate monocyte ROS production, we used the ROS-reactive dye DHR which is converted to cationic (trapping it intracellularly), green fluorescent rhodamine 123 upon oxidation and can be detected by flow cytometry. Monocytes were preincubated at 37 °C for 30 min with 10 μM DHR before being stimulated with LPS for 1 h. Inhibitors were added with either DHR (DPI) or with LPS (NAC). Following stimulation, monocytes were washed, stained with fluorophore-conjugated anti-human CD14 mAb, and analyzed by flow cytometry. ROS quantification is reported as DHR MFI for CD14+ cells.

Electrophoresis and Western Blotting—Samples were resolved on NuPAGE 4–12% BisTris gels using the Xcell Surelock™ Mini-cell system (Invitrogen) and transferred to PVDF membranes using the iBlot® Dry Blotting System (Invitrogen). After probing with biotinylated GSAO (GSAO-B), labeled proteins were detected with streptavidin-horseradish peroxidase (HRP) (Cell Signaling Technology) and visualized by enhanced chemiluminescence (Cell Signaling Technology) using a Syngene G:Box and Gene Tools 4.02(b) software (Syngene, Cambridge, UK).

Statistics—Results are reported as the mean of repeated experiments ± S.D. of the mean. Statistical analyses were performed either by one-way analysis of variance with Bonferroni tests or paired t tests, using SPSS 14.00 (Chicago, IL). Significance was attributed at the 5% level.

RESULTS

LPS Induces Expression-independent Up-regulation of Cell Surface TACE Activity—TACE activity was quantified by monitoring the rate of increase in fluorescent signal due to cleavage of the FAM-TAMRA peptide with a TACE-specific TNF sequence (27). Although previously described in cell lines, we confirmed in primary human monocytes that the rate of peptide cleavage was stable and proportional to cell number, indicating that the TACE activity assay was quantitative (Fig. 1A). To investigate the kinetics of TACE activity up-regulation, monocytes were stimulated with 1 μg/ml LPS for between 15 min and 3 h and subsequently applied to the assay. Up-regulation of TACE activity occurred after only 15-min stimulation, and we observed close to maximal up-regulation by 30 min, with a 3-fold increase in activity compared with unstimulated controls (Fig. 1B, 91 ± 16 versus 30 ± 16 fluorescence units/min at 30-min stimulation, p < 0.01). Further experiments demonstrated that LPS stimulation was dose-dependent with maximal activity up-regulation at 1 μg/ml and above (Fig. 1C). In subsequent investigations of TACE activity, monocytes were stimulated for 30 min with 1 μg/ml LPS. Cell surface TACE expression remained unchanged between treated and untreated cells throughout the time period assessed (Fig. 1, D and E, 137 ± 28 versus 136 ± 39 MFI, p = not significant). LPS-induced TACE activity up-regulation was therefore due to an increase in catalytic activity per se. This rapid up-regulation of activity with no change in expression is indicative of post-translational modification of the enzyme, potentially mediated by ROS.

ROS Mediate LPS-induced TACE Activity Up-regulation—To assess the involvement of ROS, monocytes were stimulated in the presence of 5 mM NAC, a broad spectrum free radical scavenger. NAC completely attenuated LPS-induced up-regulation of TACE activity (Fig. 2A, 84 ± 13% attenuation, p < 0.001), while having no effect on basal activity. We confirmed that LPS stimulation induced increased ROS production by preloading monocytes with the redox-sensitive fluorescent dye, DHR. Flow cytometric analysis of cells stimulated with LPS for 1 h revealed a significant increase in rhodamine fluorescence over untreated controls (Fig. 2B, 2737 ± 386 versus 1762 ± 146 MFI, p < 0.001), indicating ROS generation. LPS stimulation for 1 h, rather than 30 min, was necessary to optimize the signal: noise ratio in ROS measurement, although increased ROS could be detected after just 30-min LPS treatment (data not shown). The presence of NAC during stimulation effectively abolished LPS-induced rhodamine fluorescence (Fig. 2B, 99 ± 24% attenuation, p < 0.001), confirming the ROS-scavenging efficacy of NAC.
LPS-induced ROS Production by Flavoprotein Oxidoreductases Mediates Up-regulation of TACE Activity

The production of superoxide (O$_2^-$) by flavoprotein oxidoreductases, such as NADPH oxidase, is one of the major intracellular pathways of ROS generation (delineated in Fig. 3). Pretreatment of monocytes with 20 μM DPI, a flavoprotein oxidoreductase inhibitor, attenuated TACE activity up-regulation following LPS stimulation while having no effect on basal activity (Fig. 4A, 70% ± 6%, p < 0.05). DPI also inhibited LPS-induced ROS production (Fig. 4B).

**FIGURE 1.** Kinetics and dose response of LPS-induced TACE activity up-regulation. A, TACE-specific FRET peptide cleavage by monocytes over 60 min. Monocytes were plated at 0.5 × 10$^5$ (squares), 1 × 10$^5$ (diamonds), or 2 × 10$^5$ (triangles) cells/well with 5 μM FRET peptide. Control (circles) indicates peptide alone. The rate of peptide cleavage was stable and proportional to cell number (n = 3). B, monocytes stimulated with 1 μg/ml LPS for between 15 min and 3 h. TACE activity was up-regulated by 15 min and was close to maximal by 30 min (*, p < 0.05 versus control, n = 4; error bars, S.D.). C, monocytes were preloaded with the redox-sensitive dye DHR before 1-h LPS stimulation and subsequent flow cytometric analysis of DHR fluorescence. LPS induced up-regulation of ROS production which could be abrogated by either NAC or DPI (*, p < 0.001 different from each other, n = 4–7).

**FIGURE 2.** LPS-induced TACE activity is mediated by ROS. A, monocytes were stimulated with LPS (1 μg/ml) for 30 min in the presence or absence of NAC (5 mM). NAC completely attenuated LPS-induced TACE activity up-regulation (*, p < 0.001 different from each other, n = 4; error bars, S.D.). B, monocytes were preloaded with the redox-sensitive dye DHR before 1-h LPS stimulation and subsequent flow cytometric analysis of DHR fluorescence. LPS induced up-regulation of ROS production which could be abrogated by either NAC or DPI (*, p < 0.001 different from each other, n = 4–7).

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$10^6$ (triangles) cells/well with 5 μM FRET peptide. Control (circles) indicates peptide alone. The rate of peptide cleavage was stable and proportional to cell number (n = 3). B, monocytes stimulated with 1 μg/ml LPS for between 15 min and 3 h. TACE activity was up-regulated by 15 min and was close to maximal by 30 min (*, p < 0.05 versus control, n = 4; error bars, S.D.). C, monocytes stimulated for 30 min with concentrations of LPS from 100 pg/ml to 10 μg/ml (0.1–10,000 ng/ml). TACE activity was significantly up-regulated at 100 ng/ml LPS with maximal up-regulation at 1 μg/ml (1,000 ng/ml) and above (*, p < 0.01 versus 0 pg/ml LPS, n = 5). D, TACE surface expression on monocytes, quantified by flow cytometry, following 30-min LPS (1 μg/ml) treatment. There was no change in TACE expression on LPS treated versus control untreated monocytes (n = 4). E, representative histogram of monocyte cell surface TACE expression following 30-min LPS stimulation.
Regulation of TACE Activity in Primary Human Monocytes

FIGURE 3. Schematic of intracellular ROS production and the components of the proposed ROS-p38-MK2 TACE axis. The superoxide and nitric oxide pathways are two major pathways of intracellular ROS and RNS production, respectively. Flavoprotein oxidoreductases produce superoxide \( \left( \text{O}_2^- \right) \) which is rapidly dismutated by superoxide dismutase to \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) is then reduced to the highly reactive hydroxyl radical \( \left( \text{O}_2^- \right) \) in the \( \text{Fe}^{2+} \)-catalyzed Fenton reaction. NOS produces NO, which in turn combines with \( \text{O}_2^- \) to form highly reactive peroxynitrite \( (\text{ONOO}_2^-) \). Using the pharmacological inhibitors shown we implicated the superoxide pathway and the production of hydroxyl radicals in the up-regulation of TACE activity by LPS. Further experiments demonstrated that p38 and MK2 are also required for LPS-induced TACE activity up-regulation, and we propose that hydroxyl radicals effect the sequential phosphorylation and activation of p38 MAPK and downstream MK2, ultimately resulting in up-regulation of TACE catalytic activity.

In vivo, superoxide is dismutated by superoxide dismutase to produce \( \text{H}_2\text{O}_2 \). We questioned whether superoxide directly, or rather one of its downstream products, mediates TACE activity up-regulation. Co-incubation of cells with LPS and 5 \( \mu \text{M} \) MnPyP, a cell-permeable superoxide dismutase mimetic, had no effect on LPS-induced TACE activity up-regulation (Fig. 4A), indicating that the superoxide anion was not directly involved. Downstream of superoxide, \( \text{H}_2\text{O}_2 \) is not particularly reactive, but its \( \text{Fe}^{2+} \)-catalyzed reduction (the Fenton reaction) yields the extremely reactive hydroxyl radical \( \left( \text{O}^- \right) \). Treatment of monocytes with 5 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) induced a significant, 4-fold increase in TACE activity (Fig. 4C, 104 \pm 3 versus 26 \pm 10 fluorescence units/min, \( p < 0.001 \)), similar to that observed with LPS. Inhibition of \( \text{O}^- \) production by pretreating monocytes with 10 \( \mu \text{M} \) DFO, an \( \text{Fe}^{2+} \) chelator, caused a significant reduction in LPS-induced TACE activity up-regulation (Fig. 4D, 63 \pm 15\%, \( p < 0.001 \)). These data suggest that \( \text{H}_2\text{O}_2/\text{O}^- \), rather than superoxide, is the principal ROS involved.

Up-regulation of TACE Activity Requires ROS-mediated p38 MAPK and MK2 Activation—LPS-induced innate immune responses have been found to require ROS-mediated activation of p38 MAPK (20), prompting us to investigate whether p38 was involved in TACE activity up-regulation. Pretreatment of monocytes with 10 \( \mu \text{M} \) SB203580, a selective p38 inhibitor (30), completely abolished LPS-stimulated up-regulation of TACE activity (Fig. 5A, 97 \pm 13\%, \( p < 0.001 \)). Given the potential for pharmacological inhibitors to have off-target effects, we confirmed the involvement of the p38 MAPK pathway using two further inhibitors: SB202190, which is in the same class as SB203580 (pyridyl imidazoles); and EO 1428, a novel, highly specific inhibitor of p38 of the aminobenzophenone class (31). Both 10 \( \mu \text{M} \) SB202190 (Fig. 5B, 71 \pm 25\%, \( p < 0.001 \)) and 1 \( \mu \text{M} \) EO 1428 (Fig. 5C, 85 \pm 13\%, \( p < 0.001 \)) markedly attenuated LPS-induced TACE activity up-regulation. Increased phosphor-p38 expression over control confirmed that LPS stimulation resulted in activation of p38 (Fig. 6, A and B, 335 \pm 135 versus 15 \pm 10 MFI, \( p < 0.01 \)). The presence of the antioxidant NAC significantly attenuated LPS-induced p38 activation (Fig. 6, A and B, 48 \pm 10\%, \( p < 0.05 \)), suggesting that ROS play a role in p38 MAPK pathway in primary human monocytes. Treatment of monocytes with \( \text{H}_2\text{O}_2 \) also caused activation of p38 (Fig. 6C), and p38 inhibition prevented \( \text{H}_2\text{O}_2 \)-induced TACE activity up-regulation (Fig. 6D, 78 \pm 10\%, \( p < 0.001 \)), further suggesting that ROS act indirectly, via p38.

The ERK pathway is another redox-sensitive MAPK pathway (32). Despite a substantial reduction (79 \pm 8\%, \( p < 0.05 \)) of LPS-induced phospho-ERK by NAC treatment (Fig. 7A), inhibition of ERK with the selective ERK pathway inhibitor U0126 (33) at various concentrations resulted in only a small reduction of TACE activity up-regulation. 10 \( \mu \text{M} \) U0126 produced the greatest attenuation (Fig. 7A, 39 \pm 11\%, \( p < 0.05 \)) whereas a further increase in concentration to 20 \( \mu \text{M} \) had no significant effect (Fig. 7A, 18 \pm 11\%, \( p = \text{not significant} \)). MK2 is the principal target of p38 kinase activity and has been demonstrated to play a key role in the cellular inflammatory response (34). As expected, LPS stimulation resulted in phosphorylation MK2 (Fig. 7C), which was partially inhibited by NAC treatment (41 \pm 5\%, \( p < 0.05 \)). LPS-induced TACE activity up-regulation was almost completely attenuated by 20 \( \mu \text{M} \) MK2 Inhibitor III (35) (Fig. 7C, 87 \pm 16\%, \( p < 0.001 \)). Taken together, these data suggest that LPS-induced TACE activity up-regulation depends specifically upon the p38 MAPK-MK2 axis, a major component of which is ROS-dependent.

Cell Surface Oxidoreductase Activity Is Required for LPS-induced TACE Activity Up-regulation—Up-regulation of TACE activity has been demonstrated to be independent of the cytoplasmic domain (6, 36). Consequently, we considered other mechanisms by which the p38-MK2 axis could modify TACE enzymatic activity. Recent evidence suggests that TACE activity may be regulated by oxidation/reduction of critical disulfide bonds within the extracellular domain (37, 38). This mechanism is termed “disulfide exchange” and is typically facilitated by cell surface oxidoreductase enzymes, such as protein disulfide isomerase or thioredoxin (39). Oxidoreductases are characterized by the presence of vicinal dihioths in a CXXC motif within their active sites. GSAO is a membrane-impermeable compound that binds specifically to vicinal dihioths and can therefore inhibit oxidoreductase activity (28). LPS stimulation of monocytes in the presence of 4.5 \( \mu \text{M} \) GSAO resulted in near complete abrogation of TACE activity up-regulation (Fig. 8A,
TACE itself is known to possess two CXCC motifs and could be a direct target of GSAO. However, we demonstrated that a biotinylated form of GSAO (GSAO-B) does not bind to recombinant human TACE (rhTACE) under reducing conditions (Fig. 8B). This suggests that GSAO prevents TACE activity up-regulation indirectly, by inhibiting cell surface oxidoreductases. Finally, we confirmed that GSAO acts downstream of p38 as treatment of cells with GSAO had no effect on LPS-induced p38 phosphorylation (Fig. 8C). These results are consistent with the notion that stimulation-induced activity up-regulation requires oxidoreductase-facilitated disulfide bond rearrangement of the TACE extracellular domain.

DISCUSSION

Using a cell-based fluorometric assay, we investigated the mechanisms underlying the regulation of TACE activity in freshly isolated primary human monocytes, with particular focus on ROS and the p38 MAPK pathway. We demonstrated that LPS induces a rapid up-regulation of TACE catalytic activity without changing cell surface TACE expression. Experiments with pharmacological inhibitors provide evidence that ROS, produced by flavoprotein oxidoreductases, mediate this up-regulation by effecting the activation of the p38 MAPK pathway.

It has been firmly established that PMA stimulation of monocytic cell lines up-regulates TACE activity (14, 15, 27, 36). In contrast, there are few data concerning the more physiologically relevant LPS stimulation of primary cells. We found that LPS stimulation of primary human monocytes resulted in a significant up-regulation of TACE activity, which was maximal at an early time point (30 min) when using a high concentration (1 μg/ml) of LPS. In a previous study using the same fluorometric assay, we found using a lower 10 g/ml concentration of LPS that maximal TACE activity up-regulation in elutriated human monocytes was delayed until 2 h (40). It should be noted that in vitro the magnitude and kinetics of monocyte responses at any given LPS concentration may not necessarily reflect the in vivo situation, where modulation by additional environment-related factors is likely. In agreement with our previous study, we found that unstimulated monocytes possessed some base-line TACE activity. Other investigators have also reported basal shedding of TACE substrates, including TNF peptides (41, 42), L-selectin (43–45), and TNF receptor 1 (46). The question remains as to whether this can be ascribed to constitutive activity per se or the effects of isolation and handling of cells resulting in general cell activation and TACE activity up-regulation.

Following LPS stimulation there was no change in cell surface TACE expression within the time period assessed (up to 98 ± 16%, p < 0.001).
3 h). Such rapid, expression-independent up-regulation of TACE activity is consistent with post-translational modification of the enzyme. Scavenging of ROS, or preventing their production by flavoprotein oxidoreductases, inhibited LPS-induced TACE activity up-regulation (*, p < 0.001 different from each other, n = 4; error bars, S.D.). Similar results were obtained following pretreatment with two other p38 MAPK inhibitors, 10 μM SB202190 (B; *, p < 0.001 different from each other, n = 5) and 1 μM EO 1428 (C; *, p < 0.001 different from each other, n = 5–6). Treatment of monocytes with EO 1428 alone resulted in a modest reduction of TACE activity compared with unstimulated cells though this was not significant (p = 0.063).

FIGURE 5. p38 MAPK is required for up-regulation of TACE activity by LPS. Monocytes were pretreated with the p38 MAPK inhibitor SB203580 (10 μM) before LPS stimulation. SB203580 completely abolished LPS-induced TACE activity up-regulation (A; *, p < 0.001 different from each other, n = 4; error bars, S.D.). Similar results were obtained following pretreatment with two other p38 MAPK inhibitors, 10 μM SB202190 (B; *, p < 0.001 different from each other, n = 5) and 1 μM EO 1428 (C; *, p < 0.001 different from each other, n = 5–6). Treatment of monocytes with EO 1428 alone resulted in a modest reduction of TACE activity compared with unstimulated cells though this was not significant (p = 0.063).

FIGURE 6. ROS effect the phosphorylation and activation of p38 MAPK. A, monocytes were stimulated with LPS before flow cytometric analysis of p38 phosphorylation. LPS stimulation resulted in an increase in phospho-p38 expression at 15 min which was significantly attenuated in the presence of 5 mM NAC (*, p < 0.05 different from each other, n = 4; error bars, S.D.). B, representative histogram demonstrates increased phospho-p38 expression in LPS-treated (solid black line) versus control untreated (gray fill) monocytes and attenuation of p38 phosphorylation by NAC (dotted line). C, treatment of monocytes with H2O2 increased phospho-p38 expression. D, monocytes were preincubated with SB203580 before treatment with H2O2. SB203580 inhibited H2O2-induced TACE activity up-regulation (*, p < 0.001 different from each other, n = 4).
duced TACE activity up-regulation. This is the first time that ROS have been demonstrated to mediate an increase in TACE catalytic activity in primary cells. We used the redox-sensitive dye DHR to quantify intracellular ROS and confirm that the pharmacological agents (NAC and DPI) attenuated ROS levels. DHR fluorescence in unstimulated cells was probably due to some basal ROS production by mitochondria.

We specifically implicated the superoxide pathway (Fig. 3), rather than the NO pathway, because NOS inhibition had no effect on LPS-induced TACE activity up-regulation, a finding supported in a recent study by Bzowska et al. (47). The precise oxidoreductase(s) involved have yet to be identified: both mitochondria (48) and NADPH oxidase 4 (49) may produce ROS following LPS stimulation. Further dissection of the superoxide pathway revealed \( \text{H}_2\text{O}_2/\text{O}^\cdot \) downstream of superoxide, to be the key effector. Our data are in accord with other studies showing that inhibition of \( \text{O}^\cdot \) production attenuates the shedding of TACE substrates (50, 51). Zhang et al. previously implicated ROS/RNS in the PMA-induced up-regulation of TACE activity in the immature monocyte cell line, MonoMac 6 (14, 15). In contrast to our study, they suggested a direct role for both superoxide and nitric oxide. The disparities between our data and those of Zhang et al. likely reflect the differences between their PMA stimulation/cell line system and our more physiologically oriented system. Furthermore, Zhang and colleagues used substrate shedding as an indirect measure of TACE activity; this approach has the potential to confound as substrate modification (52) or availability (53) may influence their cleavage.

ROS have been proposed to act directly on the cysteine switch of the TACE prodomain, nullifying its inhibitory effect (15). However, the physiological applicability of this hypothesis is questionable: first, primary cells do not express the prodomain at the cell surface (2); and second, the cysteine switch may not be necessary for the inhibitory action of the prodomain (54). ROS-induced activation of p38 MAPK has been shown to be essential for LPS-induced immune responses (20). Consequently, we hypothesized that ROS may enhance TACE activity indirectly via the p38 MAPK pathway. Both LPS- and \( \text{H}_2\text{O}_2^- \)-induced TACE activity up-regulation were prevented by p38 inhibition. We confirmed the involvement of the p38 MAPK pathway using three different inhibitors of two structural classes. EO 1428 is one of a novel generation of extremely specific and potent p38 inhibitors whose selectivity for p38 has been ratified against a panel of 60 different kinases at both 1 \( \mu \text{M} \) (the concentration used in the present study) and 10 \( \mu \text{M} \) (31). Consistent with this role for p38 in regulation of TACE activity, we found that induction of phospho-p38 in monocytes by recombinant TNF was also accompanied by a significant

NAC pretreatment (dotted line) compared with unstimulated monocytes (gray fill). A, representative histogram of LPS-induced phospho-ERK (solid black line) expression at 15 min and its inhibition by MK2 Inhibitor III (20 \( \mu \text{M} \)) prior to LPS stimulation. MK2 Inhibitor III inhibited LPS-induced TACE activity up-regulation (*, \( p < 0.01 \) different from each other, \( n = 5 \)).
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**FIGURE 8.** Cell surface oxidoreductase activity is required for LPS-induced TACE activity up-regulation. A, monocytes were preincubated with GSAO (4.5 mM), a cell-impermeable inhibitor of oxidoreductase enzymes, before LPS stimulation. GSAO almost completely attenuates TACE activity up-regulation (*, p < 0.001 different from each other, n = 3; error bars, S.D.). B, BSA (5 μM, negative control, lanes 1 and 2), rhTACE (5 μM, lanes 3 and 4) or recombinant human thioredoxin (Trx) (5 μM, positive control, lanes 5 and 6) were incubated with DTT (10 μM) at room temperature for 60 min to ensure that all exposed disulfides were in the reduced form. Biotin-labeled GSAO (GSAO-B, 100 μM) was then added with (lanes 1–3) or without (lanes 2, 4, and 6) an excess of dimercaptopropanol (400 μM) for 30 min at room temperature. Dimercaptopropanol contains dithiols and preferentially sequesters GSAO, preventing it from interacting with other dithiols and ensuring that any observed protein binding is dithiol-specific. The reaction mixtures were resolved on 4–12% SDS-PAGE, transferred to PVDF, and blotted with streptavidin-HRP to detect any bound GSAO-B. In contrast to rh-Trx, GSAO-B did not bind to rhTACE. Ponceau S staining was used to confirm the presence of proteins on the membrane (data not shown). C, p38 phosphorylation was similar between monocytes stimulated with LPS alone (solid black line) and those preincubated with GSAO before LPS treatment (dotted line).

In an attempt to define our inhibitor-based findings further, we transfected primary human monocytes with a dominant negative form of p38, as described previously (55). However, we found that the short term culture (2–5 days) required for transfection resulted in maturation of monocytes to a more macrophage-like phenotype and the concomitant loss of TACE activity up-regulation in response to LPS (data not shown). The mechanisms underlying this functional change are unclear; however, it is consistent with a recent study demonstrating the absence of LPS-inducible TACE activity in cultured mouse peritoneal macrophages (41). This finding highlights the constraints of working with primary cells and reinforces the notion that TACE behavior may diverge significantly from the in vivo phenotype in in vitro maintained monocyctic cell lines. Genetic manipulation of any sort necessitates a period of culture, fundamentally altering the system we are trying to investigate. Under these limitations, the pharmacological approach we have pursued offers the most robust method to dissect the pathways regulating TACE activity.

p38 activation was found to be redox-sensitive as H₂O₂ caused p38 phosphorylation and NAC significantly reduced LPS-induced phospho-p38 expression. It is noteworthy that although both NAC and SB203580 resulted in near complete attenuation of TACE activity up-regulation, NAC reduced p38 phosphorylation by ~50%, and higher concentrations of NAC did not result in any further attenuation of p38 phosphorylation (data not shown). This incomplete abrogation by NAC is in accordance with other studies (20, 21), where p38 phosphorylation was quantified by Western blotting, and suggests that there may be a threshold below which p38 pathway activation is insufficient to produce significant TACE activity up-regulation. Alternatively, NAC, a broad spectrum free radical scavenger, may exert additional inhibitory effects via other redox-sensitive targets downstream or independent of p38 activation. Both Brill et al. and Killock et al. have recently demonstrated that p38 MAPK is necessary for L-selectin cleavage from platelets and leukocytes, respectively (24, 26). Other authors have implicated ROS/RNS in TACE substrate shedding (14, 15, 18, 19). However, this is the first time that the crucial relationship between the two pathways in mediating the up-regulation of TACE catalytic activity has been specifically addressed. The mechanisms by which ROS activate the p38 MAPK pathway have been elucidated and involve the redox-sensitive activation of apoptosis signal-regulating kinase, a MAPKKK (56, 57).

ERK, another redox-sensitive MAPK family member, has been shown to mediate PMA-induced shedding of TACE substrates (58–60). We found that inhibition of the ERK pathway had some effect on TACE activity up-regulation. However, in contrast to the complete attenuation observed with p38 inhibition, attenuation of TACE activity up-regulation by ERK pathway inhibition was small and not very robust because it did not follow a clear dose-response pattern. Interpretation of these data is not straightforward, but it certainly suggests that the involvement of the ERK pathway is minor at best. The primary target of p38, particularly in relation to post-transcriptional regulation of inflammation, is MK2 (34). Inhibition of MK2
activity with a recently developed specific inhibitor (MK2 Inhibitor III) (35) attenuated LPS-induced TACE activity up-regulation to almost the same degree as p38 inhibition. These data provide additional evidence of a specific role for activated p38 in TACE regulation. Involvement of MK2 in the regulation of TACE activity has not previously been appreciated, and it is intriguing to note that the p38-MK2 pathway is also responsible for regulating TNF biosynthesis (34). Collectively, these data demonstrate that LPS-induced ROS (or exogenous ROS in the form of H$_2$O$_2$) do not up-regulate TACE activity directly but rather do so via the specific activation of the intracellular p38 MAPK-MK2 pathway.

Although the TACE cytoplasmic domain can undergo phosphorylation (26, 58, 59) this does not appear to be involved in activity regulation as deletion of the cytoplasmic domain has no effect on stimulation-induced TACE activity up-regulation (6, 36). Current hypotheses suggest modification of the extracellular domain by disulfide exchange (37, 38). No studies have investigated the role of this mechanism in regulating TACE activity in primary human cells in response to physiological stimuli. We found that inhibition of cell surface oxidoreductases prevented LPS-induced TACE activity up-regulation.

Integrin αIIb β3 has endogenous thiol oxidoreductase activity that is believed to catalyze its own activation (61, 62). Interestingly, TACE contains two CXXC motifs, and it has been hypothesized that TACE may possess endogenous oxidoreductase activity capable of autoregulation (37). However, GSAO-B, a specific vicinal dithiol probe, did not bind to rhTACE, suggesting that the CXXC motifs are not well exposed and therefore unlikely to participate in disulfide exchange reactions. Our data favor modification of TACE structure and consequent regulation of activity, by an exogenous oxidoreductase on the cell surface, such as protein disulfide isomerase or thioredoxin. TACE contains three intramolecular disulfide bonds, and analysis with software developed by Schmidt et al. (63, 64) demonstrates that the Cys365-Cys469 bond is exposed and under strain, rendering it amenable to reduction. The finding that TACE activity up-regulation is critically dependent both on extracellular oxidoreductase activity and upstream p38-MK2 activation indicates a possible role for this signaling pathway in regulation of other cell surface disulfide exchange-mediated processes.

In summary, the present study has addressed deficiencies in previous investigations of TACE activity, namely, a reliance on substrate shedding as a surrogate for TACE activity quantification and the use of nonphysiological stimulation of monocyte cell lines. Both ROS and p38 MAPK had been independently implicated in TACE substrate shedding. Using a direct, cell-based assay of TACE catalytic activity, we have unified and enhanced these two paradigms by defining the ROS-p38-MK2-TACE axis in primary human monocytes (Fig. 3). In addition, we presented the first evidence in primary cells that thiol oxidoreductase enzymes regulate TACE activity by disulfide exchange.

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