REACTIVE OXYGEN SPECIES AND p38 MAPK MEDIATE TNF-ALPHA CONVERTING ENZYME (TACE/ADAM17) ACTIVATION IN PRIMARY HUMAN MONOCYTES.

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TNF-alpha converting enzyme (TACE) is responsible for the shedding of cell-surface TNF. Studies suggest that reactive oxygen species (ROS) mediate upregulation of TACE activity by direct oxidation or modification of the protein. However, these investigations have been largely based upon non-physiological stimulation of pro-monocytic 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 fluorimetric assay to investigate the regulation of TACE catalytic activity on freshly isolated primary human monocytes during LPS stimulation. We hypothesized that ROS mediate upregulation of TACE activity indirectly, by activation of intracellular signaling pathways.

LPS upregulated TACE activity rapidly (within 30min) without changing cell-surface TACE expression. Scavenging of ROS or inhibiting their production by flavoprotein oxidoreductases significantly attenuated LPS-induced TACE activity upregulation. Exogenous ROS (H2O2) also upregulated TACE activity with similar kinetics and magnitude as LPS. H2O2- and LPS-induced TACE activity upregulation 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 (MK2), completely attenuated TACE activity upregulation, while inhibition of ERK had little effect. Lastly, inhibition of cell-surface oxidoreductases prevented TACE activity upregulation distal to p38 activation. In conclusion, our data indicate that in primary human monocytes, ROS mediate LPS-induced upregulation 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). TNF-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 non-physiological 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 upregulation in physiological states.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the upregulation 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 upregulation 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 as 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 upregulation ‘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 fluorimetric 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 in order to preserve, as far as possible, their in vivo phenotype. We identified the crucial role of ROS in mediating LPS-induced TACE activity upregulation in human monocytes. Further investigation revealed that ROS mediate TACE activity upregulation 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 HBSS were purchased from Invitrogen (Paisley, UK). Sigma Chemical Company (Dorset, UK) supplied the following: saponin, DMSO, BSA, desferrioxamine (DFO), diphenyleneiodonium chloride (DPI), EDTA, H₂O₂, Manganese(III) 5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine chloride tetrakis (MnPyP), N-acetyl-cysteine (NAC, BioXtra grade), SB203580, SB202190, U0126, dithiothreitol (DTT), 2,3-dimercaptopropanol (DMP), and Ponceau S staining solution. N-Nitro-L-Arginine Methyl Ester hydrochloride (L-NAME) and MK2 Inhibitor III were from Merck (Darmstadt, Germany). LPS (E. 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–SPLAQAVRSSSRK–TAMRA) (27).

R&D systems (Abingdon, UK) supplied fluorophore-conjugated anti-human TACE ectodomain mAb and relevant isotype control, while BD Biosciences (Oxford, UK) supplied anti-human CD14 mAb and 7-amino actinomycin D (7-AAD). Fluorophore conjugated anti-human phospho-p38, phospho-ERK and phospho-MK2 mAbs were from Cell Signaling Technology (Danvers, USA).

Cell harvest and purification- 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 manufacturer’s instructions. PBMCs were washed and resuspended in separation buffer (HBSS supplemented with 0.1% BSA and 2mM EDTA). CD14+ monocytes were purified from PBMCs by negative immunomagnetic bead separation according to manufacturer’s (Miltenyi Biotech, Surrey, UK) instructions. Briefly, non-monocytes were labeled with a cocktail 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 over 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 HBSS supplemented with 5% FCS at a concentration of 5×10^6 cells/ml. The kinetics and dose-dependency of LPS stimulation were assessed with incubation times varying between 15min and 3h, and increasing LPS concentrations from 100pg/ml to 10µg/ml. To investigate the role of ROS, monocytes were pre-incubated with NAC (neutral pH adjusted), MnPyP, DPI, DFO or L-NAME for 30min before 30min LPS stimulation. In addition, cells were stimulated with H_2O_2. MAPK pathways were investigated by pre-treating 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 pre-treated with 4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide (GSAO), a membrane-impermeable, specific, vicinal thiol blocker (28), for 30min. Concentrations of all pharmacological agents used (described in the results section) are considered to be ‘saturating doses’, consistent with those found within the relevant literature. DMSO 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 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 10min at room temperature, before being washed and resuspended in FACS wash buffer (FWB –PBS supplemented with 2% FCS, 5mM 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 manufacturer’s instructions. Cells were then washed twice in perm/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 30min at room temperature in the dark, before a further wash and resuspension in perm/wash buffer. Analysis of TACE and phospho-p38 expression was performed with a CyAn ADP Analyzer flow cytometer (Beckman Coulter, Bucks, UK) and Flowjo software (Tree Star, Ashland, OR, USA). 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-AAD to samples prior to acquisition according to manufacturer’s instructions.

**TACE Activity Assay-** Following stimulation, monocytes were washed and resuspended in assay buffer (HBSS supplemented with 1% BSA). Monocyte number and viability were assessed as described above before being plated at 1×10^5 cells/well in black 96 well plates (Corning Inc., Surrey, UK). Cells were plated in duplicate or, number permitting, triplicate. Well volumes totaled 100µl with 5µM 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 fluorimeter (Flx-800, Bio-tek Instruments Inc., Bedfordshire, UK) at 37°C and at λ_{ex} 485nm and λ_{em} 535nm 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 approximately 5min, enzymatic activity was calculated as the gradient of the fluorescence-time plot over a 10min period, giving values in fluorescence units/min (FU/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 pre-incubated at 37°C for 30min with 10µM DHR before being stimulated with LPS for 1h. 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% Bis-Tris 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 visualised 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 ± SD 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, Illinois, USA). Significance was attributed at the 5% level.

RESULTS

LPS induces expression-independent upregulation 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 upregulation, monocytes were stimulated with 1µg/ml LPS for between 15min and 3h and subsequently applied to the assay. Upregulation of TACE activity occurred after only 15min stimulation and we observed close to maximal upregulation by 30min, with a 3-fold increase in activity compared to unstimulated controls (Fig 1B: 91±16 vs. 30±16 FU/min at 30min stimulation, p<0.01). Further experiments demonstrated that LPS stimulation was dose-dependent with maximal activity upregulation at 1µg/ml and above (Fig 1C). In subsequent investigations of TACE activity, monocytes were stimulated for 30min with 1µg/ml LPS. Cell-surface TACE expression remained unchanged between treated and untreated cells throughout the time period assessed (Figs 1D and E: 137±28 vs. 136±39 MFI, p=not significant). LPS-induced TACE activity upregulation was therefore due to an increase in catalytic activity per se. This rapid upregulation of activity with no change in expression is indicative of post-translational modification of the enzyme, potentially mediated by ROS.

Reactive oxygen species mediate LPS-induced TACE activity upregulation. To assess the involvement of ROS, monocytes were stimulated in the presence of 5mM NAC, a broad spectrum free radical scavenger. NAC completely attenuated LPS-induced upregulation of TACE activity (Fig 2A: 84±13% attenuation, p<0.001), whilst having no effect on basal activity. We confirmed that LPS stimulation induced increased ROS production by pre-loading monocytes with the redox-sensitive fluorescent dye, DHR. Flow cytometric analysis of cells stimulated with LPS for 1h revealed a significant increase in rhodamine fluorescence over untreated controls (Fig 2B: 2737±386 vs. 1762±146 MFI, p<0.001), indicating ROS generation. LPS stimulation for 1h, rather than 30min, was necessary to optimise the signal:noise ratio in ROS measurement, although increased ROS could be detected after just 30min 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 upregulation of TACE activity. The production of superoxide (O₂⁻) by flavoprotein oxidoreductases, such as NADPH oxidase, is one of the major intracellular pathways of ROS generation (delineated in Figure 3). Pre-treatment of monocytes with 20µM DPI, a flavoprotein oxidoreductase inhibitor, attenuated TACE activity upregulation
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 2B: 139±38%, p<0.001). Nitric oxide (NO) production by nitric oxide synthase (NOS) constitutes another pathway of radical production. As DPI has been shown to inhibit NOS (29), we confirmed that its mechanism of action in the present study was limited to oxidoreductase inhibition: pre-treatment of monocytes with 10mM L-NAME, a NOS inhibitor, had no effect on LPS-induced TACE activity upregulation (Fig 4A).

In vivo, superoxide is dismutated by superoxide dismutase (SOD) to produce H₂O₂. We questioned whether superoxide directly, or rather one of its downstream products, mediates TACE activity upregulation. Co-incubation of cells with LPS and 5µM MnPyP, a cell-permeable SOD mimic, had no effect on LPS-induced TACE activity upregulation (Fig 4B), indicating that the superoxide anion was not directly involved. Downstream of superoxide, H₂O₂ is not particularly reactive but its Fe²⁺-catalysed reduction (the Fenton reaction) yields the extremely reactive hydroxyl radical (·OH). Treatment of monocytes with 5mM H₂O₂ induced a significant, 4-fold increase in TACE activity (Fig 4C: 104±3 vs. 26±10FU/min, p<0.001), similar to that observed with LPS. Inhibition of ·OH production by pre-treating monocytes with 10mM DFO, an Fe²⁺ chelator, caused a significant reduction in LPS-induced TACE activity upregulation (Fig 4D: 63±15%, p<0.01). These data suggest that H₂O₂/·OH, rather than superoxide, is the principal ROS involved.

Upregulation 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 upregulation. Pre-treatment of monocytes with 10µM SB203580, a selective p38 inhibitor (30), completely abolished LPS-stimulated upregulation of TACE activity (Fig 5A: 97±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 (pyridinyl imidazoles); and EO1428, a novel, highly specific inhibitor of p38 of the aminobenzophenone class (31). Both 10µM SB202190 (Fig 5B: 71±25%, p<0.001) and 1µM EO1428 (Fig 5C: 85±13%, p<0.001) markedly attenuated LPS-induced TACE activity upregulation. Increased phospho-p38 expression over control confirmed that LPS stimulation resulted in activation of p38 (Figs 6A and B: 335±135 vs. 15±10 MFI, p<0.01). The presence of the antioxidant NAC significantly attenuated LPS-induced p38 activation (Figs 6A and B: 48±10%, p<0.05), suggesting that ROS play a role in p38 MAPK pathway in primary human monocytes. Treatment of monocytes with H₂O₂ also caused activation of p38 (Fig 6C) and p38 inhibition prevented H₂O₂-induced TACE activity upregulation (Fig 6D: 78±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±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 upregulation. 10µM U0126 produced the greatest attenuation (Fig 7A: 39±11%, p<0.05) while a further increase in concentration to 20µM had no significant effect (Fig 7A: 18±11%, p=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±5%, p<0.05). LPS-induced TACE activity upregulation was almost completely attenuated by 20µM MK2 inhibitor III (35) (Fig 7C: 87±16%, p<0.001). Taken together, these data suggest that LPS-induced TACE activity upregulation 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 upregulation. Upregulation 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 disulphide isomerase (PDI) or thioredoxin (Trx) (39). Oxidoreductases are characterized by the presence of vicinal dithiols in a CXXC motif within their active sites. GSAO is a membrane-impermeable compound that binds specifically to vicinal dithiols and can therefore inhibit oxidoreductase activity (28). LPS-stimulation of monocytes in the presence of 4.5mM GSAO resulted in near complete abrogation of TACE activity upregulation (Fig 8A: 98±16%, p<0.001). TACE itself is known to possess two CXXC 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 upregulation 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 upregulation requires oxidoreductase-facilitated disulfide bond rearrangement of the TACE extracellular domain.

**DISCUSSION**

Using a cell-based fluorimetric assay this study 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 demonstrate that LPS induces a rapid upregulation of TACE catalytic activity without changing cell-surface TACE expression. Experiments with pharmacological inhibitors provide evidence that ROS, produced by flavoprotein oxidoreductases, mediate this upregulation by effecting the activation of the p38 MAPK pathway.

It has been firmly established that PMA stimulation of monocyte cell lines upregulates TACE activity (14,15,27,36). In contrast, there is little data concerning the more physiologically-relevant LPS stimulation of primary cells. We found that LPS stimulation of primary human monocytes resulted in a significant upregulation of TACE activity, which was maximal at an early time-point (30min) when using a high concentration (1µg/ml) of LPS. In a previous study using the same fluorimetric assay, we found using a lower 10g/ml concentration of LPS that maximal TACE activity upregulation in elutriated human monocytes was delayed until 2h (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 baseline 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 upregulation.

Following LPS stimulation there was no change in cell-surface TACE expression within the time period assessed (up to 3h). Such rapid, expression-independent upregulation 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 upregulation. 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 nitric oxide pathway, as NOS inhibition had no effect on LPS-induced TACE activity upregulation, 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 H₂O₂/OH⁻, downstream of superoxide, to be the key effector. Our data are in accord with other studies showing that inhibition of OH⁻ production attenuates the shedding of TACE substrates (50,51). Zhang et al. previously implicated ROS/RNS in the PMA-induced upregulation 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 that of Zhang et al. likely reflect the differences between their PMA stimulation/cell line system and our more physiologically-orientated 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 pro-domain, nullifying its inhibitory effect (15). However, the physiological applicability of this hypothesis is questionable: firstly, primary cells do not express the prodomain at the cell surface (2) and secondly, the cysteine switch may not be necessary for the inhibitory action of the pro-domain (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 H2O2-induced TACE activity upregulation 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 1µM (the concentration used in the present study) and 10µM (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 increase (~2.5-fold at 1h) in their TACE activity (data not shown).

In an attempt to further define our inhibitor based findings, we transfected primary human monocytes with a dominant negative form of p38, as previously described (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 upregulation 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 while both NAC and SB203580 resulted in near complete attenuation of TACE activity upregulation, 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 upregulation. 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 upregulation 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 upregulation. However, in contrast to the complete attenuation observed with p38 inhibition, attenuation of TACE activity upregulation by ERK pathway inhibition was small and not very robust as it did not follow a clear dose-response pattern. Interpretation of this
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 upregulation to almost the same degree as p38 inhibition. This data provides 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 H₂O₂) do not upregulate 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 upregulation (6,36). Current hypotheses suggest modification of the extracellular domain by disulphide 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 upregulation.

Integrin αIIb β3 has endogenous thiol oxidoreductase activity that is believed to catalyse 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 disulphide exchange reactions. Our data favor modification of TACE structure and consequent regulation of activity, by an exogenous oxidoreductase on the cell-surface, such as PDI or Trx. TACE contains three intramolecular disulphide 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 upregulation is critically dependent 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 non-physiological 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 disulphide exchange.

**REFERENCES**


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The abbreviations used are: DFO, desferrioxamine; DHR, dihydrorhodamine 123; DTT, dithiothreitol; DMP, 2,3-dimercaptopropanol; DPI, diphenyleneiodonium; GSAO, (4-(N-(S-glutathionyl)acetyl)amino)phenylarsenoxide); GSAO-B, (4-(N-(S-(6-(N-(6-(N-(6-((biotinoyl)amino)hexanoyl)amino)hexanoyl)glutathionyl)-acetyl)amino)phenylarsenoxide); HRP, horseradish peroxidase; L-NAME, Nitro-L-Arginine Methyl Ester hydrochloride; MFI, mean fluorescence intensity; MK2, MAPK-activated protein kinase 2; MnPyP, Manganese(III) 5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine chloride tetrakis; NAC, N-acetylcysteine; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TACE, TNF-alpha converting enzyme.

**FIGURE LEGENDS**

**Figure 1:** Kinetics and dose-response of LPS-induced TACE activity upregulation.

A, TACE-specific FRET peptide cleavage by monocytes over 60min. Monocytes were plated at either 0.5×10⁵ (squares), 1×10⁵ (diamonds) or 2×10⁶ (triangles) cells/well with 5µM FRET peptide. Control (circles) indicates peptide alone. The rate of peptide cleavage was stable and proportional to cell number (n=3). B, monocytes were stimulated with 1µg/ml LPS for between 15min and 3h. TACE activity was upregulated by 15min and was close to maximal by 30min (*p<0.05 vs. control, n=4). C, monocytes were stimulated for 30min with concentrations of LPS from 100pg/ml to 10µg/ml (0.1 to 10,000ng/ml). TACE activity was significantly upregulated at 100ng/ml LPS with maximal upregulation at 1µg/ml (1000ng/ml) and above (*p<0.01 vs. 0pg/ml LPS, n=5). D, TACE surface expression on monocytes, quantified by flow cytometry, following 30min LPS (1µg/ml) treatment. There was no change in TACE expression on LPS treated vs. control untreated monocytes (n=4). E, representative histogram of monocyte cell-surface TACE expression following 30min LPS stimulation.

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

**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 (O₂⁻) which is rapidly dismutated by superoxide dismutase (SOD) to H₂O₂. H₂O₂ is then reduced to the highly reactive hydroxyl radical (·OH) in the Fe²⁺-catalysed Fenton reaction. Nitric oxide synthase
(NOS) produces nitric oxide (NO) which in turn combines with $O_2^-$ to form highly reactive peroxynitrite (ONOO$^-)$.

Using the pharmacological inhibitors shown we implicated the superoxide pathway and the production of hydroxyl radicals in the upregulation of TACE activity by LPS. Further experiments demonstrated that p38 and MK2 are also required for LPS-induced TACE activity upregulation and we propose that hydroxyl radicals effect the sequential phosphorylation and activation of p38 MAPK and down-stream MK2, ultimately resulting in upregulation of TACE catalytic activity.

Figure 4: Flavoprotein oxidoreductase-derived ROS mediate LPS-induced TACE activity upregulation. A, monocytes were pre-treated with either DPI (20µM) or L-NAME (10mM) before LPS stimulation. DPI caused significant attenuation of LPS-induced TACE activity upregulation while L-NAME had no effect (*p<0.05 different from each other, n=5). B, LPS stimulation of monocytes in the presence of MnPyP (5µM) had no significant effect on TACE activity upregulation (n=4). C, treatment of monocytes for 30min with H$_2$O$_2$ (5mM) resulted in upregulation of TACE activity similar to that seen with LPS (*p<0.001 vs. control, n=4). D, monocytes were pre-treated with DFO (10mM) before LPS stimulation. DFO abrogated TACE activity upregulation by LPS (*p<0.01 different from each other, n=4).

Figure 5: p38 MAPK is required for upregulation of TACE activity by LPS. Monocytes were pre-treated with the p38 MAPK inhibitor SB203580 (10µM) before LPS stimulation. SB203580 completely abolished LPS-induced TACE activity upregulation (A, *p<0.001 different from each other, n=4). Similar results were obtained following pre-treatment with two other p38 MAPK inhibitors, 10µM SB202190 (B, *p<0.001 different from each other, n=5) and 1µM 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 to 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 15min which was significantly attenuated in the presence of 5mM NAC (*p<0.05 different from each other, n=4). B, representative histogram demonstrating increased phospho-p38 expression in LPS treated (black line) vs. control untreated (gray fill) monocytes and attenuation of p38 phosphorylation by NAC (dotted line). C, treatment of monocytes with H$_2$O$_2$ increased phospho-p38 expression. D, monocytes were pre-incubated with SB203580 before treatment with H$_2$O$_2$. SB203580 inhibited H$_2$O$_2$-induced TACE activity upregulation (*p<0.001 different from each other, n=4).

Figure 7: MK2 mediates upregulation of TACE activity while the ERK pathway has only a minimal role. A, representative histogram of LPS-induced phospho-ERK (black line) expression at 15min and its inhibition by NAC pre-treatment (dotted line) in comparison to unstimulated monocytes (gray fill) B, monocytes were pre-incubated with U0126 at concentrations of 1, 10 and 20µM before being treated with LPS. We observed significant reduction of TACE activity upregulation with 10µM U0126, but not 1 or 20µM (*p<0.05 different from each other, n=3-5). C, representative histogram of LPS-induced phospho-MK2 (black line) expression at 15min and its inhibition by NAC pre-treatment (dotted line) in comparison to unstimulated monocytes (gray fill) D, monocytes were pre-treated with MK2 Inhibitor III (20µM) prior to LPS stimulation. MK2 Inhibitor III inhibited LPS-induced TACE activity upregulation (*p<0.001 different from each other, n=5).

Figure 8: Cell-surface oxidoreductase activity is required for LPS-induced TACE activity upregulation. A, Monocytes were pre-incubated with GSAO (4.5mM), a cell-impermeable inhibitor of oxidoreductase enzymes, before LPS stimulation. GSAO almost completely attenuated TACE activity upregulation (*p<0.001 different from each other, n=3). B, BSA (5µM, negative control, lanes 1 and 2), rhTACE (5µM, lanes 3 and 4) or rhTrx (5µM, positive control, lanes 5 and 6) were incubated with DTT (10µM) at room temperature for 60min to ensure that all exposed disulfides were in the reduced form. Biotin labeled GSAO (GSAO-B, 100µM) was then added with (lanes 1, 2 and 3) or without (lanes 2, 4 and 6) an excess of dimercaptopropanol (DMP, 400µM) for 30min at room temperature.
DMP 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 rhTrx, 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 line) and those pre-incubated with GSAO before LPS treatment (dotted line).