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LPS activates ADAM9 dependent shedding of ACE from endothelial cells

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ABSTRACT

Angiotensin-I converting enzyme (ACE) is a zinc dependent peptidase with a major role in regulating vasoactive peptide metabolism. ACE, a transmembrane protein, undergoes proteolysis, or shedding, by an as yet unidentified proteinase to release a catalytically active soluble form of the enzyme. Physiologically, soluble ACE in plasma is derived primarily from endothelial cells. We demonstrate that ACE shedding from confluent endothelial cells is increased in response to bacterial lipopolysaccharide, but not phorbol esters. Characterisation of lipopolysaccharide stimulated shedding showed that there is a lag phase before soluble ACE can be detected which is sensitive to inhibitors of translation, NF-κB, TNFα and TNFR-I/II. The shedding phase is less sensitive to these inhibitors, but is ablated by BB-94, a Matrix Metalloproteinase (MMP)/A Disintegrin and Metalloproteinase (ADAM) inhibitor. Tissue Inhibitor of Metalloproteinase (TIMP) profiling suggested a requirement for ADAM9 in lipopolysaccharide induced ACE shedding, which was confirmed by depletion with siRNA. Transient transfection of ADAM9 and ACE cDNAs into HEK293 cells demonstrated that ADAM9 requires both membrane anchorage and its catalytic domain to shed ACE.

1. Introduction

ACE (ACE, EC 3.4.15.1) is a zinc dependent metalloproteinase that cleaves the decapeptide angiotensin-I (Ang-I) to the potent vasopressor angiotensin-II (Ang-II) and inactivates bradykinin, a potent vasodilatory peptide, regulating fluid homeostasis, electrolyte balance and blood pressure in mammals [1–3]. Somatic ACE is a 140 kDa peptide (150–180 kDa glycosylated) with two catalytic centres within the ectodomain linked to a stalk region followed by a transmembrane and intracellular domain [2,4]. ACE can undergo proteolysis within the stalk region, a process referred to as shedding, which results in release of a soluble active form from the cell surface. Plasma ACE results from this process and is derived primarily from endothelial cells [5–7]. Investigations using genetically engineered mice have indicated that the membrane anchored form (tissue ACE), rather than plasma ACE is principally involved in regulating vasotension [8]. Expression of ACE exclusively in hepatocytes or endothelial cells, but not in kidney proximal tubule epithelial cells, restores this deficiency in ACE null mice [9,10]. The membrane bound and soluble forms of ACE also convert Ang-I to Ang-II with differing abilities at the two catalytic sites [11]. These findings suggest that shedding may provide a means of down regulating tissue ACE function and/or alter substrate turnover and selectivity. It has been shown that endotoxin induced lung endothelial injury and oedema is accompanied by an increase in plasma ACE and a decrease in tissue ACE providing evidence for a role in ACE shedding in pathology [12].

Although there has been biochemical characterisation of the basic properties of ACE secretase, or sheddase, activity, as yet ACE secretase has not been identified. It is known that ACE shedding is primarily metalloproteinase dependent and can be blocked by inhibitors of the MMP or ADAM families. ACE secretase is similar to Amyloid Precursor Protein (APP) α-secretase (ADAM9, 10, 17 [13]), and is not the TNFα converting enzyme (TACE, ADAM17) [13–15]. In this article we demonstrate bacterial lipopolysaccharide (LPS) induces shedding of ACE from endothelial cells and characterise the mechanism of action.

2. Materials and methods

2.1. Materials

Cryo-preserved pooled donor HUVEC at passage 2 or proliferating pooled donor HUVEC at passage 1 (p1) were purchased from TCS Cellworks (UK). Large vessel endothelial growth medium,

Abbreviations: IL-1, Interleukin-1; TNFα, Tumour Necrosis Factor alpha; Fc, Fc portion of IgG; siRNA, short interfering RNA; PMA, phorbol 12-myristate 13-acetate; Mca, (7-methoxycourmarin-4-yl)acetyl; Dpa, N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; ICAM-1, Intracellular Adhesion Molecule-1; VCAM-1, Vascular Cell Adhesion Molecule-1.

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phenol red free, with growth supplements (2% Foetal Calf Serum (FCS), EGF, Heparin, hydrocortisone, bFGF), was purchased from TCS Cellworks (UK). HEK293 cells were obtained from Cancer Research UK. FCS was purchased from Hyclone. The ACE N-domain selective substrate Mca-ASDK-Dpa-OH, (provided by C.G. Knight, University of Cambridge Dept. Biochemistry) was synthesised as described previously [16,17]. Penicillin, streptomycin, LPS from *E. coli* 055:B5, emetine and PMA were from Sigma (UK). Mouse-anti human ACE (clone 9B9, MAB4051) was from Chemicon. Control mouse IgG_1 was purchased from Dako, UK. Rabbit anti-human ADAM9 intracellular domain antibody (A9/369) was raised to the peptide SSQGNLIPARPAPAP for Western blot analysis. EZ-link™ NHS–LC–Biotin was purchased from Thermofisher, UK. BB-94 was a kind gift from British Biotech, UK. TIMP-1, -2 and -3 were prepared as described previously [18]. Function blocking antibodies to TNFα, TNFR-I and TNFR-II [19] and recombinant human TNFα were purchased from R & D Systems Europe. SN50 and control peptides for the inhibition of nuclear translocation of NF-κB [20] were purchased from Merck. ELISAs for human TNFα and VCAM-1 were purchased from Immuno-Diagnostic Systems (UK).

2.2. Endothelial cell culture and stimulation by PMA and LPS

HUVEC at p1 in T25 flasks were cultured to confluency in the large vessel endothelial cell culture medium supplied at 37 °C and 5% CO_2. HUVEC p2 were cultured on plastic and to a maximum of p5. Cells were seeded to confluency at p4–5 into 6 or 12 well tissue culture plates. HUVEC were stimulated with either 25 ng ml^{-1} PMA (40 pM) or 2 μg ml^{-1} LPS for various time points up to 16 h.

2.3. Biotinylation and immunoprecipitation of ACE from HUVEC

To detect endogenous ACE from HUVEC the cells were labelled with biotin. HUVEC were cultured to confluency in either T25 flasks (p1) or 10 cm tissue culture dishes (p5) before washing in serum free medium (SFM). Cells were labelled at 37 °C with 50 μg ml^{-1} EZ-link NHS–LC–Biotin for 15 min in SFM. The cells were then washed in SFM, 100 mM glycine for 5 min to block reactive biotin. Finally, cells were incubated in fresh medium for 10 min before covering with a minimal volume and stimulating with LPS or PMA in the presence or absence of 10 μM BB-94. After 5 h, medium was removed, centrifuged at 13,000×*g* for 5 min and pre-cleared of IgG by incubating for 2 h with 60 μl 50% v/v protein-G slurry. After centrifugation at 300×*g* for 5 min the supernatant was subjected to IP with the monoclonal anti-human ACE (5 μg ml^{-1}) and 60 μl 50% v/v protein-G slurry per ml sample over night at 4 °C and washed 2× with SFM. Protein-G beads were boiled in 2× reducing Laemmli buffer and protein resolved on 7% reducing SDS–PAGE. Biotinylated ACE was revealed using streptavadin–HRP and enhanced chemi-luminescence (ECL) after transfer to nitrocellulose.

2.4. Analysis of ACE activity in cell medium

Cell debris was removed from medium by centrifugation at 13,000×*g* for 5 min prior to analysis. ACE activity was monitored in medium diluted 4:1 to result in a final concentration of 50 mM HEPES, 200 mM KCl, 100 μM $ZnSO_4$, pH8.0, 20 μM Mca-ASDK-Dpa-OH and Complete™ EDTA-free protease inhibitor cocktail (Roche GmbH). Changes in fluorescence were measured with respect to time in 96 well black microtitre plates in a TECAN spectrofluor plus microtitre plate reader equipped with 320 nm excitation and 390 nm emission cut-off filters.

2.5. Inhibition of ACE secretion from HUVEC

Confluent HUVEC p5, in 6 or 12 well plates were incubated with 250 nM TIMP-1, -2 or -3, 10 μM BB-94, 5 μM emetine, 25 μM NF-κB or control inhibitory peptide, or 2 μg ml^{-1} IgG, anti-TNFα, TNFR-I or TNFR-II. Inhibitors were added to the medium 30 min before the addition of LPS or 2.5 h after LPS. Cells were then cultured for 16 h following LPS stimulation at which point the medium was harvested and analysed for ACE activity.

2.6. Transfection of HUVEC with siRNA targeting ADAM9

Custom ADAM9 duplexes targeted to two regions of the mRNA (A9Hu1: AAGAGCCUCUGAAAUGUGGAG, A9Hu2: AAUGUACAAGAGAUACCUGUA), were purchased from Dharmacon. A pool of 4 non-specific control duplexes was purchased from Qiagen. Although ADAM9 could be readily depleted at the mRNA level (unpublished data), depletion of ADAM9 protein required two siRNA transfections and higher levels of depletion were found using the two oligos in combination; HUVEC at approximately 70–80% confluency, p4 were transfected overnight with Oligofectamine (Invitrogen, UK) and both siRNA in 6 well plates for 24 h. siRNA was transfected into HUVEC again at p5 for 24 h before stimulation of ACE shedding when the cell monolayer had reached confluency. Down regulation of ADAM9 was quantified by densitometry of

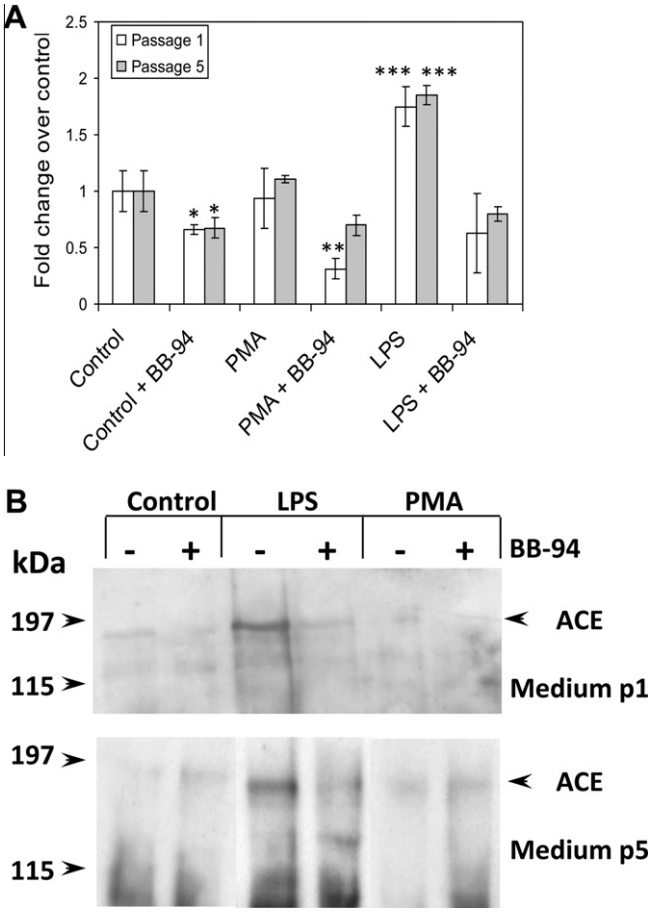

Fig. 1. ACE shedding from HUVEC. Confluent HUVEC were stimulated with 25 ng ml^{-1} PMA or 2 μg ml^{-1} LPS for 16 h in the presence or absence of 10 μM BB-94. A. ACE activity in the medium of cells at passage 1 and 5, Data are the mean ± stdev, $n = 3$ and p values were determined using ANOVA vs. Control values. B. Immuno-precipitation of ACE from the medium of cells labelled with biotin and stimulated with PMA or LPS with or without 10 μM BB-94 for 5 h and by Western blot. The full blot of medium at p5 is shown in the supplementary Fig. 2A.

western blots using the Bio Rad Quantity One™ software and normalised for the endothelial marker protein CD31.

2.7. Transfection of human ACE and ADAM9 cDNAs into HEK293

Human somatic ACE cDNA was subcloned from pbACE2 [21] into pCDNA3.1 zeo+. cDNAs to mouse ADAM9, were prepared as described previously [22] or were a generous gift from CelltechChiroscience (Slough, UK). HEK293 were cultured in DMEM, 10% FCS, glutamine, penicillin, streptomycin. Cells were removed from the culture plastic by washing in fresh medium and resuspended after centrifugation to a density of 1×10^6 ml^{-1}. The cells were transfected with a Fugene-6 (Roche Diagnostics GmbH), 1 μg ACE cDNA and either 1 μg pCDNA3.1 zeo+ or 1 μg metalloproteinase cDNA with 3 μl Fugene-6 for 10 min. The transfected cells were then seeded into 6 well plates at confluency. 16 h after transfection the cell medium was replaced with phenol red free DMEM with 2% FCS (v/v) before culturing for 24 h. Cell medium was prepared and analysed for ACE activity as described previously. Cell lysates were prepared by washing the cells twice with cold PBS before lysing in 50 mM Tris–HCl, 1% NP-40 and Complete™ EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH). Lysates were centrifuged at 13,000×*g* for 5 min to remove debris before Western blotting and detection by ECL.

2.8. Statistical analysis of data

In experiments where a comparison between multiple variables was made, data was analysed using one-way ANOVA with a Bonferroni post-test. Where pair wise comparisons were made, a

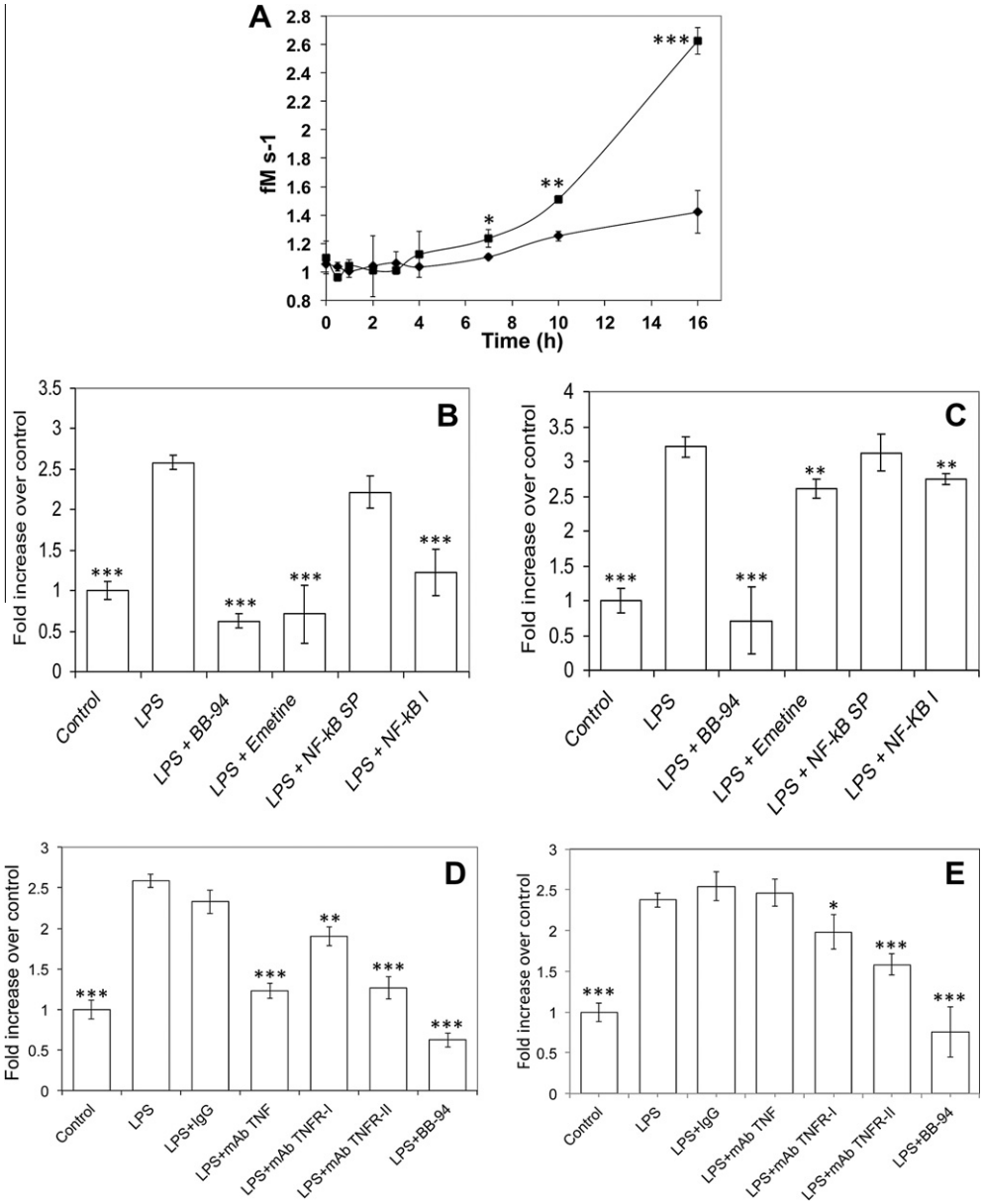

Fig. 2. Kinetics and Inhibition of ACE shedding from HUVEC. A. Confluent HUVEC stimulated with and without 2 μg/ml LPS and ACE activity measured in the culture medium at the times inidicated. Data are the mean ± stdev, $n = 3$. B and C. Confluent HUVEC were stimulated with 2 μg ml^{-1} LPS 30 min after the addition of 5 μM emetine, 25 μM control or NF-kB SN50 peptides, 2 μg ml^{-1} isotype control IgG or inhibitory antibodies. Medium was analysed for ACE activity 16 h after addition of LPS. D and E. HUVECs were inhibited as described in B/C, but the inhibitors were added 2.5 h after LPS. Medium was analysed for ACE activity 16 h after LPS addition. Data are the mean ± stdev, $n = 3$ and p values indicated are vs. LPS treated samples.

Student's *T*-Test with a Bonferroni correction was used. Analysis was performed using GraphPad Prism version 5.0c for Mac OSX, GraphPad Software, San Diego California USA, www.graphpad.com. *, ** and *** are used in the figures to indicate $p < 0.05$, 0.001 and 0.0001, respectively.

3. Results

PMA induces the shedding of a number of proteins from endothelial cells including TNFα, VCAM-1 and Tie-1 [23,24]. LPS has been shown to stimulate shedding of TNFα [25] and ACE *in vivo* [12]. In order to establish if ACE could be shed from HUVEC, we stimulated confluent HUVEC with PMA and LPS. Using an ACE specific substrate selective for the N-terminal active site, Mca-ASDK-Dpa-OH we found that ACE was shed into the medium of HUVEC up to passage 5 on stimulation with LPS. Shedding of ACE was inhibited by the broad-spectrum metalloproteinase inhibitor BB-94, indicating MMP/ADAM activity was required (Fig. 1A). PMA treatment of cells did not increase shedding of ACE (Fig. 1A), although VCAM-1 was shed into the medium as detected by ELISA (Supplementary data Fig. 1). In order to detect shed ACE by western blot, we subjected the confluent HUVEC monolayers to cell surface biotinylation before stimulating shedding with LPS. Upon immuno-precipitation a band of approximately 180 kDa was detected, which was increased in the presence of LPS and was inhibited by BB-94 (Fig. 1B).

We next examined the kinetics of shedding of ACE from confluent HUVEC in response to LPS. In contrast to shedding by PMA in cell lines, which is typically rapid (<30 min), LPS induced shedding showed a lag phase of approximately 3–4 h before an increase in ACE over basal levels could be detected (Fig. 2A). LPS has been to shown induce transcription of a number of pro-inflammatory molecules, including TNFα via NF-κB [26]. In order to demonstrate that ACE shedding was dependent on NF-κB, a cell permeable peptide capable of inhibiting NF-κB nuclear translocation, SN50 [20] was added 30 min before the addition of LPS or at 2.5 h after the addition of LPS to determine the relative dependence of the shedding and lag phases on NF-κB. Shedding was inhibited when the NF-κB inhibitor was added before LPS (Fig. 2B), but not after 2.5 h indicating the lag phase involves NF-κB signalling (Fig. 2C). Emetine, an inhibitor of the 40s ribosome subunit, inhibited shedding in the same manner (Fig. 2B, C).

LPS has been shown to transiently activate the transcription of TNFα leading to an increase in the 27 kDa membrane bound form of TNFα (mTNFα [28]). An inhibitory antibody to both soluble and mTNFα added at 30 min prior or at 2.5 h after the addition of LPS, was only effective when added 30 min before LPS (Fig. 2B, C). mTNFα has been shown to signal primarily through TNFRII rather than TNFRI [27]. Inhibitory antibodies to TNFRII were more effective in inhibiting ACE shedding than those targeting TNFRI, particularly at 2.5 h after LPS, where anti-TNFRI had a reduced effect (Fig. 2B, C).

TIMP inhibition profiling of ectodomain shedding can aid identification of which MMP/ADAM is involved [28]. Basal ACE shedding was inhibited by TIMP-3, but in the presence of LPS virtually no inhibition by TIMPs was observed (Fig. 3A). Analysis of VCAM-1 in the same samples showed significant inhibition by

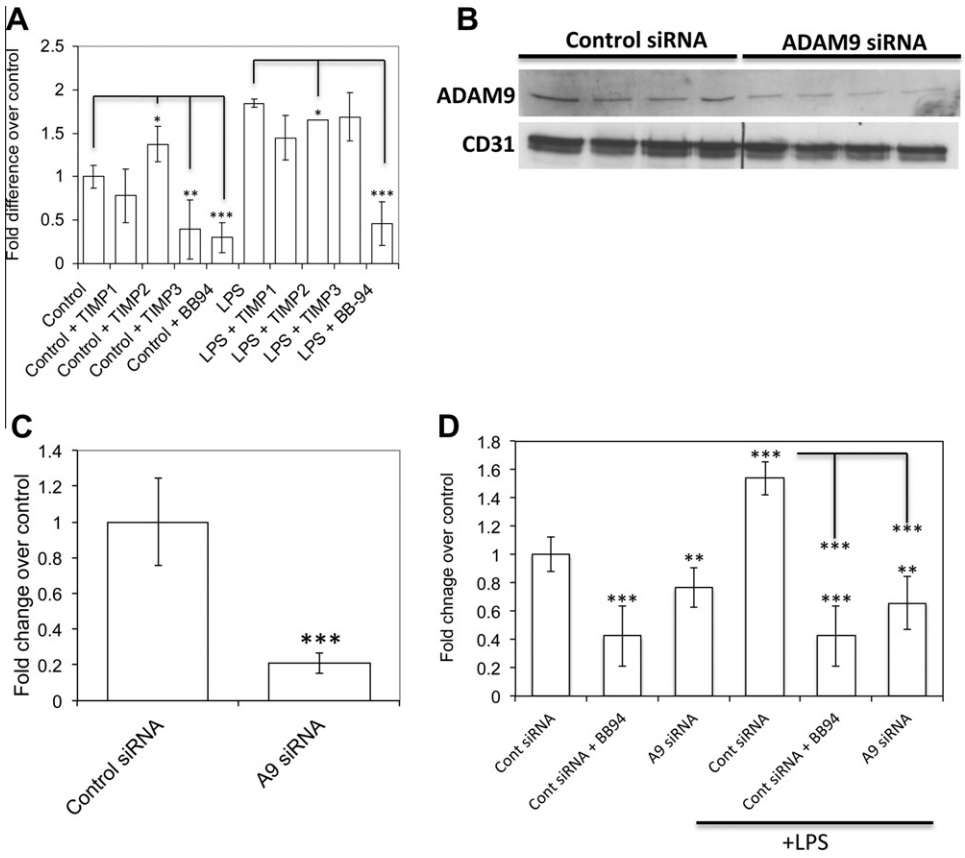

Fig. 3. TIMP inhibition and siRNA depletion show ADAM9 is required for LPS induced ACE shedding. A. HUVEC were stimulated with LPS in the presence or absence of 250 nM TIMP-1, -2 or -3 or 10 μM BB-94 for 16 h and ACE activity measured. Data are the mean ± stdev, $n = 3$, $p < 0.05$ was determined using ANOVA as indicated. B and C. HUVEC were transfected with either 100 nM control or ADAM9 siRNA. Lysates from 4 independent transfections were analysed for changes in ADAM9 by western blot and normalised against CD31 (B/C) for quantification of depletion. D. HUVEC analysed in B/C were seeded at confluence for 16 h then stimulated with LPS for 16 h before ACE levels were quantified in the medium. BB-94 (10 μM) was included as a positive control for inhibition of shedding. Data are the mean ± stdev, $n = 4$. *p* values are vs. Control values unless separately indicated.

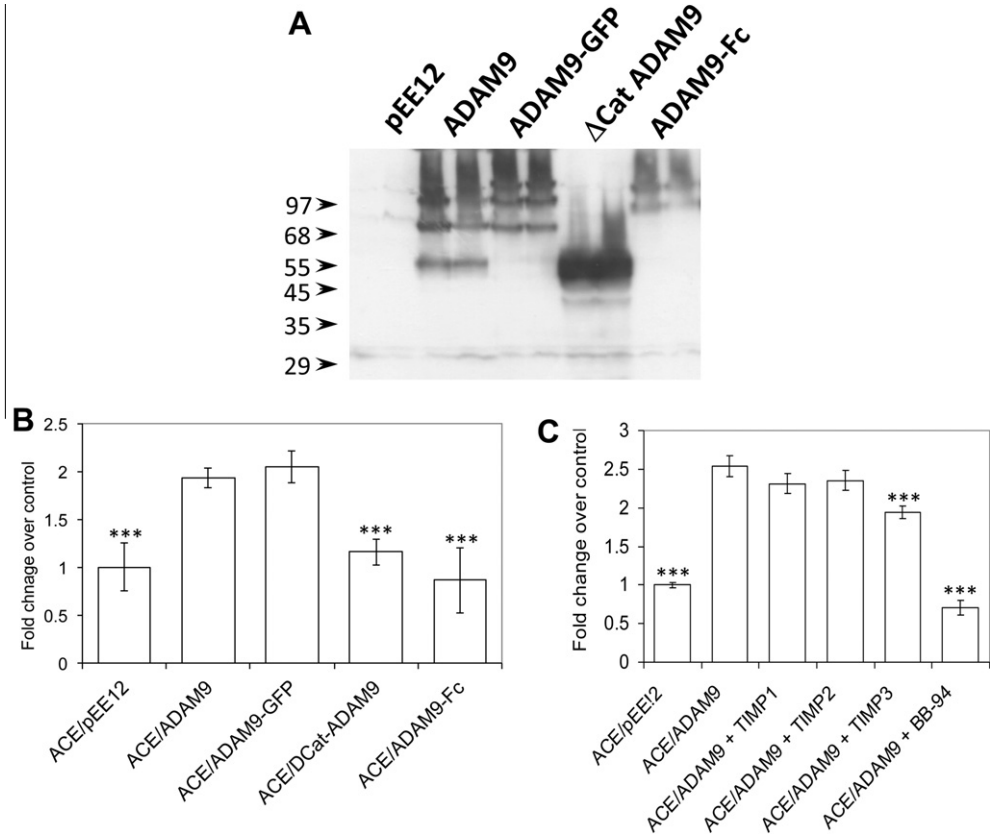

Fig. 4. ADAM9 shedding of ACE requires membrane anchorage and the catalytic domain. 293T cells were transiently transfected with either the control vectors pEE12 or cDNAs containing ADAM9, ADAM9–GFP, ADAM9–Δcat, ADAM9–Fc, and the ACE cDNA. A. Lysates were harvested after 16 h and analysed by western blot for expression ADAM9. B. 16 h after transfection, medium was changed and then analysed for ACE activity after 16 h. Data are the mean ± stdev, $n = 3$. C. ACE and either pEE12 or ADAM9 cDNA were co-10 μM) was added and ACE activity assayed after 16 h. Data are the mean ± stdev, $n = 3$. p values are vs. ACE/ADAM9 values.

TIMP-3 and to lesser extent by TIMP-2 and TIMP-1 (Supplementary data Fig. 1) in agreement with previous findings [23,29]. Hence TIMP profiling indicated involvement of ADAM8 or 9 in ACE proteolysis on LPS stimulation [22], ADAM8 mRNA is not detectable in HUVEC, whereas ADAM9 mRNA is highly abundant [30]. To show that ADAM9 was the LPS stimulated ACE secretase, HUVEC were transfected with siRNA to ADAM9. Depletion of ADAM9 by approximately 80% was confirmed by western blot and quantification of the depletion of ADAM9 was normalised against expression of CD31 (Fig. 3B, C). Basal shedding of ACE was inhibited to a degree by ADAM9 siRNA, whereas LPS stimulated shedding was significantly inhibited by ADAM9 siRNA (Fig. 3C).

To confirm that ADAM9 shedding of ACE requires the proteolytic activity of ADAM9 and membrane anchorage [31], the cDNA of ACE was transiently co-transfected into HEK293 cells with cDNAs encoding wild-type ADAM9 (ADAM9-wt), ADAM9 lacking the catalytic domain (ADAM9-Δcat) or a soluble ADAM9 IgG–Fc domain fusion (ADAM9–Fc). ADAM9 with a C-terminal EGFP fusion was used to test the importance of the C-terminal residues. ACE shedding was significantly increased on transfection of ADAM9–WT and ADAM9–GFP cDNAs, but not ADAM9-Δcat or ADAM9-Fc (Fig. 4A, B). ACE shedding by ADAM9 in HEK293 cells was also inhibited by BB-94, but not potently by TIMPs at the concentrations used in the HUVEC experiments (Fig. 4C).

4. Discussion

Although it has been established for a number of years that ACE is shed by 'ACE secretase', identified as a Zn^{2+} dependent membrane anchored proteinase, the identity of ACE secretase, or secretases, has remained elusive. In this article we show that LPS, but not PMA, can induce shedding of ACE in confluent endothelial cells with a lag phase after stimulation with LPS, observed before soluble ACE can be detected. The use of function blocking antibodies showed that TNFα and TNFRI/TNFRII were also required predominantly in the lag phase. LPS has been shown to induce the transient expression of the 27 kDa membrane form of TNFα [32], which signals through TNFRII [33], matching the period when ACE shedding is sensitive to inhibitors of TNFα and TNFRI/II. We were also unable to detect soluble TNFα by ELISA on LPS stimulation, although very low levels were detected on PMA stimulation within 30 min (unpublished data). Recombinant soluble TNFα could induce ACE shedding and was inhibited by BB-94, but differed from LPS stimulated shedding in that it was inhibited by the antibody to TNFRI, and not TNFRII, or siRNA to ADAM9. Although ACE activity could be detected in lysates, lower levels of biotinylated ACE were detected, indicating less may come from the cell surface on TNFα stimulation (Supplementary data Fig. 2). Our data indicate that ACE shedding may be mediated by mTNFα that activates ADAM9 via TNFRI/II and represents a novel signalling mechanism by which ADAM9 can be activated in endothelial cells. This also shows that ACE shedding is mediated by more than one pro-inflammatory stimulus and is activated by different proteolytic and signalling systems. ADAM9 knockout mice do not show increased levels of soluble ACE in their plasma [34] and our data shows that ADAM9 may only have a limited role in shedding in unstimulated endothelial cells *in vitro*, which suggests that ADAM9 may not play a significant role in constitutive shedding of ACE. ADAM9 could play a role in endotoxin induced lung endothelial injury [12] and may be responsible for the increase in plasma ACE.

In conclusion, bacterial LPS can induce shedding of ACE from endothelial cells that is dependent on ADAM9. The kinetics of shedding and our inhibitor studies indicate that NF-κB, translation TNFα – TNFRI/II signalling is required first before ADAM9 dependent shedding of ACE can be initiated. This is distinct from shedding induced by soluble TNFα, suggesting that membrane anchored TNFα is required. This is a novel mechanism by which ADAM9 is activated in endothelial cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.113.

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