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A role for increased mRNA stability in the induction of endothelin-1 synthesis by lipopolysaccharide

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Abstract

An association exists between infection and cardiovascular diseases, including atherosclerosis, stroke and myocardial infarction. This may involve endothelin-1 (ET-1) which has been implicated in these and other vascular pathologies. ET-1 synthesis is controlled primarily by the level of its mRNA and numerous stimuli, including infection, lead to elevated ET-1 levels. Here, we have investigated the regulation of ET-1 release and preproET-1 (ppET-1) mRNA in bovine aortic endothelial cells by lipopolysaccharide (LPS). ET-1 release from bovine aortic endothelial cells was stimulated by LPS and reporter gene assays implicated LPS-induced ppET-1 transcription. However, changes in transcription were modest compared to increases in ET-1 synthesis. Therefore, ppET-1 mRNA levels were measured by real-time reverse transcription–polymerase chain reaction. The effect of LPS on ppET-1 mRNA levels was more marked than on transcription (1.2-fold increase in transcription vs. 5.5-fold increase in ppET-1 mRNA). Analysis of ppET-1 mRNA stability by real-time reverse transcription–polymerase chain reaction showed that LPS increased its half-life by approximately 2-fold. Thus, upregulated ppET-1 mRNA and hence increased ET-1 synthesis may be due to both increased transcription and reduced mRNA degradation. These effects of LPS on mRNA stability may be a key mechanism in vascular pathologies through which many proteins are induced in response to infection.

Keywords: Endothelin-1; Preproendothelin-1; Reporter gene; mRNA stability; Lipopolysaccharide; Real-time PCR

1. Introduction

ET-1 is an endothelium-derived peptide [1] that exerts a wide range of effects on various tissues and cell types through interaction with two subtypes of G-protein-coupled receptors, ET_A and ET_B [2]. ET-1 has been implicated in the pathogenesis of cardiovascular diseases, including hypertension, congestive heart failure and atherosclerosis [2,3]. Inflammation is known to play a pivotal role in the pathogenesis of atherosclerosis [4] and accumulating epidemiological and experimental evidence links bacterial infection and cardiovascular pathology [5]. Pathogen burden has recently been shown to be an independent risk factor for the presence and severity of coronary artery disease [6]. LPS is the major source of inflammation in Gram-negative bacterial infection and genetic polymorphisms of LPS receptors are linked to cardiovascular pathology. For example, a toll-like receptor-4 polymorphism conferring a reduced inflammatory response is associated with a reduced risk of atherosclerosis [7], and conversely, a CD14 polymorphism resulting in higher CD14 expression is a risk factor for myocardial infarction [8]. Exposure of vascular endothelial cells to LPS activates an adhesive cell phenotype *via* the prototypical inflammatory transcription factor nuclear factor kappa B (NF-κB) [9] and LPS activation of NF-κB has been linked to atherosclerotic lesion development [10]. ppET-1 mRNA is induced by LPS in endothelial cells [11] and ET-1 in an inflammatory context has recently been implicated in the progression of cardiovascular disease [12].

ET-1 is produced by intracellular proteolytic processing of the 212 amino acid precursor ppET-1 [1,3,13]. Its biosynthesis and secretion are controlled by the level of ppET-1

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Abbreviations: ET-1, endothelin-1; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; ppET-1, preproendothelin-1; bp, base pairs; kb, kilobase pairs; RT, reverse transcription; PCR, polymerase chain reaction; BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MG115, carbobenzoxy-leucyl-leucyl-norvalinal; BHQ1, black hole quencher-1; C_T, cycle number to threshold.

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mRNA [3]. There is little evidence for a storage mechanism prior to release [14] and both stimulatory and inhibitory effects on ET-1 secretion are correlated with ppET-1 mRNA levels [15]. The 5′ flanking region of the human ppET-1 gene contains numerous elements known to be involved in transcriptional regulation [13,16]. Although complete homology exists between human and porcine ET-1, there is less similarity elsewhere in the ppET-1 sequence. Nevertheless, considerable homology is seen within a 250 bp section of the 3′ untranslated region of ppET-1 mRNA. This suggests that the 3′ untranslated region is conserved due to an important role in regulation of ppET-1 mRNA, for example, by altering ppET-1 mRNA transcript stability [13,16].

Information on human ppET-1 promoter regulation has been derived from a number of reporter gene studies. Regions essential for high basal levels of ppET-1 promoter activity in endothelial cells have been identified containing the GATA binding protein-2 (positions −136 to −131 relative to the transcription start site) and AP-1 (positions −109 to −102) motifs [17–19]. An element binding the vascular endothelial zinc finger-1 protein and mediating endothelial cell-specific gene expression has recently been described in the ppET-1 promoter [20]. Human ppET-1 promoter fragments of various sizes have demonstrated transcriptional activity in response to a number of stimuli, including insulin (−4.4 kb) [21], hypoxia (−669 bp) [22], advanced glycation end product (−3.6 kb) [23], fluid shear stress (−2.9 kb) [24], thrombin (−952 bp) [25], transforming growth factor-β, tumour necrosis factor-α and interleukin-1 (−2.9 kb) [26]. Such evidence points to the importance of transcriptional regulation in stimulated ET-1 secretion. Preliminary evidence for mRNA transcript stability influencing steady-state ppET-1 mRNA levels has been described in studies of transforming growth factor-β and phorbol ester [27].

A more detailed understanding of the regulation of ET-1 in the vascular endothelium by bacterial infection is important for defining the link between infection and vascular pathophysiology. Therefore, the aim of this study was to investigate the regulation of ppET-1 mRNA in endothelial cells by LPS. For this purpose, we developed human ppET-1 luciferase reporter gene assays for investigations of transcription, and a real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) system for measurement of ppET-1 mRNA levels. We have examined stimulation of ET-1 by LPS at the level of peptide release, transcriptional activity, mRNA abundance and mRNA stability.

2. Materials and methods

2.1. Human ppET-1 reporter genes

pET4.7-luc and pET1.9-luc plasmids were constructed in which expression of the luciferase gene was controlled by −4725 or −1881 to +167 bp of the human ppET-1 promoter, respectively. cDNA for these constructs (clone 451B15 (GenBank z98050) derived from the library RPCI3) was provided by The Sanger Centre. A 5.1 kb PCR fragment containing the ppET-1 promoter was produced using the forward primer 5′-GG*ACGCGT*ATCTCGGCTCACCA-CAACCTCT-3′ (containing *Mlu*I), the reverse primer 5′-AAGTCAACGAGCGTGCCTACCTG-3′ and Platinum *Pfx* DNA polymerase (Life Technologies) for 30 cycles (94°, 15 s; 55°, 30 s; 70°, 6 min). A 4892 bp *Mlu*I/*Bgl*II fragment (pET4.7-luc) and a 2047 bp *Xma*I/*Bgl*II fragment (pET1.9-luc) were cloned into pGL3-Basic (Promega), upstream of the luciferase cDNA. Plasmid identities were confirmed by automated DNA sequencing using an ABI 310 Genetic Analyzer (Applied Biosystems). pSV-β-Galactosidase and pGL3-Control vectors were obtained from Promega.

2.2. Cell culture and transient transfection

Bovine aortic endothelial cells (BAEC) were derived by clonal selection from primary cultures [15] and cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% (v/v) foetal calf serum (FCS). For transient transfection, BAEC were seeded in 12-well plates (4 cm^2 growth area per well) at an appropriate density to give approximately 70% confluence on the following day. Co-transfection of a luciferase reporter plasmid and pSV-β-Galactosidase, giving a total DNA amount of 300 ng, was performed using TransFast Reagent (Promega). Transfection was performed in serum-free DMEM for 1 hr with a transfection reagent:DNA charge ratio of 2 and complete culture medium was then added to give a final FCS concentration of 10% (v/v). Forty-eight hours after transfection, cells were used for experimentation. Untreated or transiently transfected BAEC were incubated for 6 hr with *Escherichia coli* LPS (Serotype 0127B8; Sigma) with 0.5% (v/v) FCS in DMEM (Life Technologies), with and without carbobenzoxy-leucyl-leucyl-norvalinal (MG115) (Peptide Institute). Culture media were collected for ET-1 assay and cells were lysed for preparation of cell extract and assay of reporter activity where appropriate. For immunoblotting, confluent BAEC in 25 cm^2 flasks were stimulated with LPS (1 μg/mL, 0.5% (v/v) FCS) for 15, 30 or 60 min. Cytosolic and nuclear protein extracts were prepared as described previously [28]. For ppET-1 mRNA experiments, confluent BAEC cultures were grown in 24-well plates (2 cm^2 growth area per well) and incubated with or without LPS for 1 or 3 hr prior to the addition of the RNA polymerase II inhibitor, actinomycin D (10 μg/mL; Sigma). Cells were harvested for mRNA extraction at known time intervals.

2.3. Assays

ET-1 released into the cell culture medium was measured by a 96-well plate sandwich immunoassay using an

affinity-purified rabbit anti-ET-$1_{[1–15]}$ IgG for plate coating and a biotinylated rabbit anti-ET-$1_{[16–21]}$ IgG and ^{125}I-streptavidin detection system. Cells lysates prepared in Reporter Lysis Buffer were assayed for luciferase activity using BrightGlo Reagent and β-galactosidase activity using the β-Galactosidase Assay System according to manufacturer's instructions (Promega). Our intention was to express luciferase activity relative to the control reporter β-galactosidase. However, our data showed β-galactosidase activity to be affected in a concentration-dependent manner by LPS (0, 3, 10 mg/mL LPS: 0.347 ± 0.02 (N = 3); 0.615 ± 0.04 (N = 3); 0.683 ± 0.02 (N = 3) absorbance units, respectively) and recently others have observed that such normalisation may be unreliable [29]. Transfection efficiency is important when comparing different reporter constructs, however, in these experiments comparisons are made between different cell treatments following transfection with the same DNA. Therefore, uncorrected luciferase measurements were analysed. For immunoblotting, cytosolic and nuclear proteins in equivalent amounts were resolved by 7.5% SDS–PAGE and electrotransferred to PVDF membranes (BioRad). Membranes were immunoblotted with 0.5 μg/mL affinity-purified rabbit polyclonal IgG for p65 (sc-372) (Santa Cruz Biotechnology Inc.) according to supplier's instructions. Immunoreactive proteins were visualised by enhanced chemiluminescence (Pierce) following incubation with a goat anti-rabbit IgG peroxidase-conjugated antibody (Pierce). A single band was detected, with an apparent molecular mass of 65 kDa.

2.4. RT–PCR and mRNA half-life

RNA was extracted using a total RNA miniprep kit according to manufacturer's instructions (Stratagene). Steady-state ppET-1 mRNA levels were assessed by RT and real-time PCR using Taqman methodology (Applied Biosystems). cDNA was prepared from 300 to 500 ng RNA template using avian myeloblastosis virus reverse transcriptase (7.5 U per reaction; Promega) and primed with random hexamers (0.25 μg) for 45 min at 42°. Real-time quantitative PCR for ppET-1was performed in duplicate or triplicate on each cDNA sample. 10–25 ng RNA equivalents of cDNA template was amplified using the sequence-specific primers 5′-AAGAGTGTGTCTACTTCTGCCATCTG-3′ and 5′-AAAGAAGTCCTTTAAGGAGCGCT-3′ (300 nM each) and probe 5′-FAM-TGGGTCAACACTCCAGAGCACGTTGTT-3′ in a 20 μL reaction volume. Initial experiments compared the use of probes containing either black hole quencher-1 (BHQ1) or TAMRA as the 3′ quencher. 18S rRNA was measured using ribosomal RNA control reagents (Applied Biosystems). 40 cycle PCR reactions were performed in an ABI 5700 Sequence Detection System (Applied Biosystems).

To monitor disappearance of the ppET-1 mRNA transcript and determine its half-life, mRNA levels were quantified at timed intervals following actinomycin D treatment. Under non-limiting conditions, a doubling of PCR product occurs with each cycle and an essential feature of real-time PCR is that detection of product is performed after every thermal cycle. Hence, this method can be used to determine mRNA half-life more precisely than conventional methods. Threshold fluorescence was set within the exponential phase of the amplification plot and the cycle number at which each sample reached this value was determined (cycle number to threshold (C_T) value). Regression analysis of C_T vs. sample time was used to determine the time required for an increase in C_T of 1.0 for each treatment (1/slope). This indicates a halving of cDNA template and hence represents the half-life of the mRNA transcript.

2.5. Materials

Unless otherwise stated, all chemicals and standard reagents were from Sigma or Merck and all general molecular biology reagents and enzymes were from Promega. Tissue culture medium and reagents were from Sigma or Life Technologies. Oligonucleotides were from Oswel and bovine ET-1 Taqman probes were from Oswel (TAMRA) or Cruachem Ltd (BHQ1).

2.6. Statistical analysis

Results are expressed as mean ± SEM. Data were analysed using GraphPad Prism (GraphPad Software). Statistical analysis was performed using an ANOVA with Fisher's protected least-significant difference post hoc test using Statview version 5.0.1. Statistical significance was considered as a P value of less than 0.05.

3. Results and discussion

3.1. ET-1 secretion and ppET-1 promoter activity in BAEC

Treatment of BAEC with LPS produced a concentration-dependent increase in ET-1 release (Fig. 1A) and translocation of NF-κB p65 to the nucleus (Fig. 1B). The proteasome inhibitor MG115 produced a concentration-dependent inhibition of basal and LPS-induced ET release in BAEC (Fig. 1C). In transiently transfected BAEC, basal luciferase expression from pET4.7-luc was greater than that from pET1.9-luc (32% increase, $P < 0.01$). Treatment of pET4.7-luc transfected BAEC with LPS (10 μg/mL) resulted in a significant increase in ET-1 release into the culture medium and was accompanied by a small but significant increase in human ppET-1 promoter activity (Fig. 2A and B). As would be expected, ET-1 release in pET1.9-luc transfected BAEC was similarly increased by LPS (data not shown), however, no effect of LPS on

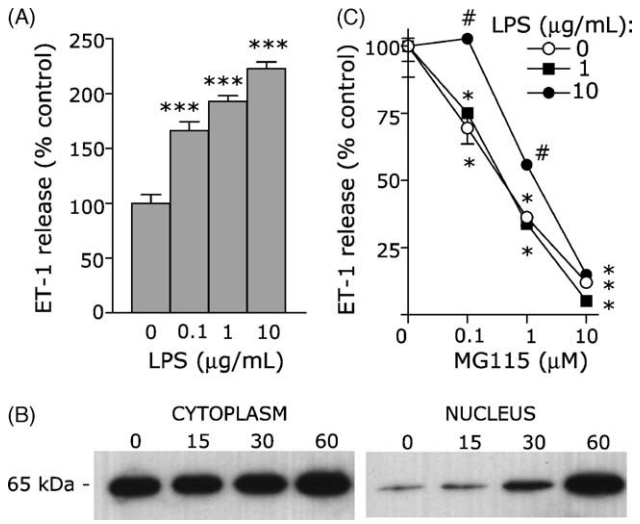

Fig. 1. (A) and (C) Effects of LPS and MG115 on ET-1 release in BAEC. BAEC were incubated with LPS (0.1–10 μg/mL) alone or in combination with MG115 (10 μM). Results are mean ± SEM (N = 3) expressed relative to control and are representative of three similar experiments. $^{*}P < 0.005$, $^{***}P < 0.001$ compared to control; $^{\#}P < 0.05$ compared to without LPS. Release of ET-1 under controlled conditions was 22.2 ± 1.9 fmol/cm^2/6 hr. (B) Effect of LPS on p65 translocation in BAEC. BAEC were incubated with LPS (1 μg/mL) for 1 hr and cytoplasmic and nuclear p65 content were measured by immunoblotting using a rabbit polyclonal IgG for p65 which gave a single band of the expected size.

promoter activity was seen (Fig. 2A). MG115 (10 μM) prevented the LPS-induced increases in ET-1 release and human ppET-1 reporter expression in pET4.7-luc transfected BAEC and also reduced basal ppET-1 reporter gene expression in these cells (Fig. 2A and B). Function of the pET1.9-luc construct was confirmed by induction of reporter expression in the presence of transforming growth factor-β (1 ng/mL) (pET1.9-luc: 142 ± 9% (N = 27), pET4.7-luc: 143 ± 6% (N = 15); $P < 0.001$ compared to corresponding controls) while transient transfection of BAEC with the promoterless luciferase vector pGL3-Basic did not induce reporter expression (data not shown).

In agreement with our observations, LPS has previously been demonstrated to induce ET-1 secretion in a concentration-dependent manner [30] and to upregulate ppET-1 mRNA expression in endothelial cells [11,31]. Here, we have demonstrated that LPS-induced ppET-1 transcription required the presence of a promoter region between 1.9 and 4.7 kb upstream of the transcription start site. This is consistent with the hypothesis that effects of LPS on ppET-1 gene transcription are mediated by NF-κB since NF-κB consensus sequences were only present in pET4.7-luc. Concentration-dependent inhibition of ET-1 release in BAEC was seen in response to the proteasome inhibitor MG115, which is commonly used to prevents nuclear translocation of NF-κB p65 [32]. Furthermore, the proteasome inhibition prevented the LPS-induced changes in ppET-1 reporter gene expression. Effects of LPS on gene expression in human endothelial cells have recently been assessed using cDNA microarrays where ET-1 was shown to be induced, and furthermore this was prevented by an inhibitor of NF-κB nuclear translocation [31]. Overexpression of NF-κB p50 and p65 has been shown to stimulate ppET-1 transcription and increased ppET-1 mRNA in endothelial cells [19], thus demonstrating NF-κB responsiveness of the ppET-1 promoter. We found a component of the basal level of ppET-1 mRNA transcription to be mediated by a region 1.9 kb upstream from the transcription start site. This may reflect an involvement of NF-κB in basal ppET-1 gene expression since proteasome inhibition also reduced basal ppET-1 reporter expression in pET4.7-luc transfected BAEC.

3.2. ppET-1 mRNA levels and mRNA stability in BAEC

A real-time PCR assay for bovine ppET-1 was developed to study mRNA levels in BAEC. To ensure this assay had the greatest sensitivity, two probes with different quencher dyes were compared; TAMRA and the more recently described BHQ1. Comparison of the amplification plots of known amounts of cDNA assayed under optimised conditions for the two probes showed both distinguished between nanogram differences in starting template. However, the BHQ1 probe provided greater sensitivity (lower

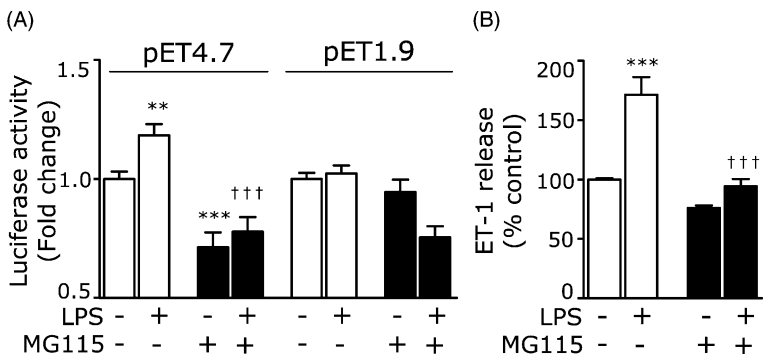

Fig. 2. Effect of LPS on human ppET-1 promoter activity and ET-1 release in transfected BAEC. BAEC were transiently transfected with pET4.7-luc or pET1.9-luc and 48 hr later were treated with LPS (10 μg/mL) and MG115 (10 μM) for 6 hr. Results are mean ± SEM of three to eight experiments performed in triplicate and expressed relative to control. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control. Release of ET-1 under controlled conditions was 31.5 ± 3.0 fmol/cm^2/6 hr and 27.7 ± 2.0 fmol/cm^2/6 hr in pET4.7-luc and pET1.9-luc transfected BAEC, respectively.

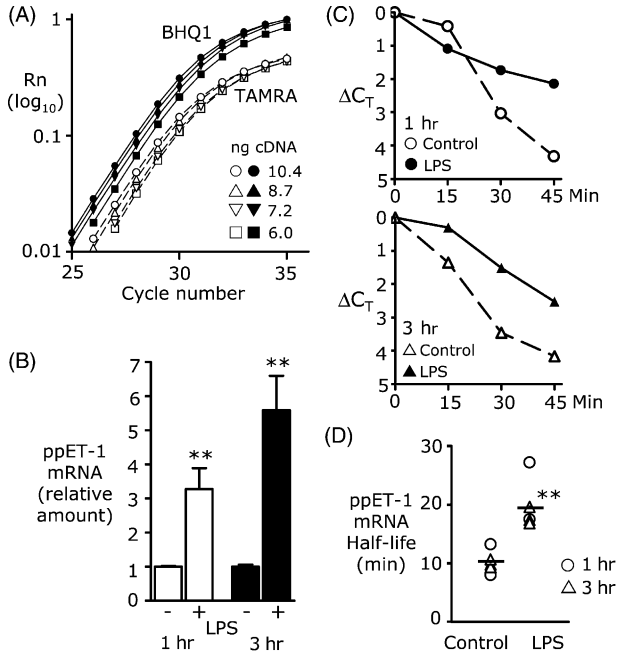

Fig. 3. Effect of LPS on ppET-1 mRNA levels and stability in BAEC. (A) Evaluation of real-time PCR assays for ppET-1. Total RNA was subjected to RT with random primers and real-time PCR using a specific primer pair and probe containing either the BHQ1 or TAMRA quencher. Amplification plots of normalised reporter signal (R_n) vs. cycle number are shown for known amounts of cDNA assayed in triplicate. Results shown are representative of an experiment performed three times. (B) Steady-state ppET-1 mRNA levels calculated as mRNA amount adjusted for 18S and expressed relative to control. BAEC were treated with LPS (10 μg/mL) and RNA was subjected to RT and real-time quantitative PCR for ppET-1 and 18S. Results are mean ± SEM (N = 3) and are representative of five similar experiments. $^{**}P < 0.01$ compared to corresponding control. (C) Plot of change in cycle number to threshold (C_T) vs. time illustrating decay of ppET-1 mRNA. BAEC were treated with LPS (10 μg/mL) for 1 or 3 hr prior to the addition of actinomycin D (10 μg/mL) and RNA harvested at 15 min intervals was subjected to RT and real-time quantitative PCR for ppET-1. Results are mean of two or three experiments (N = 6, 1 hr; N = 3, 3 hr). (D) ppET-1 mRNA half-life calculated as the time required for an increase in C_T of 1.0 (1/slope of C_T vs. time plot). Data points represent individual experiments with either 1 hr (○, N = 3) or 3 hr (△, N = 1) stimulation. Horizontal bars represent mean values (N = 5). $^{**}P < 0.0$ compared to control.

C_T values) and higher maximum signal for the same samples (Fig. 3A). Therefore, this probe was chosen for subsequent analysis by real-time PCR.

Steady-state ppET-1 mRNA levels were significantly increased by LPS after 1 and 3 hr treatment (Fig. 3B). The degree of LPS-induced ppET-1 transcription was relatively small when compared to both the increase in steady-state ppET-1 mRNA levels and the degree of induction of ET-1 peptide secretion. Transcription of mRNA is not the sole determinant of steady-state mRNA levels, but contributes to a balance in which the mRNA level is the net result of the relative rates of mRNA transcription and degradation; hence stability of mRNA represents an important factor in controlling mRNA abundance. We determined the half-life of ppET-1 mRNA in endothelial cells under basal conditions to be 10 min and demonstrated increased ppET-1 mRNA half-life following stimulation with LPS (Fig. 3C and D). There are no previous reports of ppET-1 mRNA half-life obtained using real-time PCR techniques, however, previous estimates of ppET-1 mRNA half-life based on less sensitive techniques are in the order of 15–30 min [11,16]. Our studies show that LPS stabilises ppET-1 mRNA resulting in an approximately 2-fold increase in half-life. Stability of mRNA is important for regulating the level of gene expression for many cytokines. LPS stabilises tumour necrosis factor-α mRNA *via* p38 mitogen-activated protein kinase and this pathway is linked to AU-rich element-mediated stabilisation of tumour necrosis factor-α mRNA [33]. The 3′ untranslated region of ppET-1 mRNA may be involved in regulating the stability of ppET-1 mRNA *via* AU-rich elements in a similar manner [16,34].

Co-ordinated induction of ET-1 synthesis by increased transcription and enhanced mRNA stability describes a mechanism for LPS responses that is likely to be of fundamental importance in the link between local or systemic infection and vascular pathophysiology. Increasing evidence links bacterial infection to stroke, myocardial infarction and atherosclerosis [5]. It has been suggested that ET-1 participates in end organ damage of inflammatory responses and LPS and NF-κB may be pivotal to this process [10,12]. Endothelial cells express the predominant receptor for LPS: toll-like receptor-4, which activates gene expression through NF-κB and p38 [9,35]. Therefore, a generalised sequence of events following bacterial infection may be exposure of the endothelium to LPS, toll-like receptor-4-mediated activation of NF-κB and p38, and ultimately upregulation of multiple genes, including ET-1. Stabilisation of a variety of mRNA transcripts may be a critical element in this response of the endothelium to LPS, and consequent elevation of mediators, such as ET-1, may be an underlying feature of bacterial infection contributing to associated vascular pathophysiology. More detailed elucidation of mechanisms of ET-1 activation by potentially pathological stimuli will provide information for the development and use of potential interventions in a variety of vascular diseases.

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