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Stimulatory effects of lipopolysaccharide on endothelial cell activin and follistatin

Kathryn M. Wilson [a], A. Ian Smith [b], David J. Phillips [a,*]

[a] *Monash Institute of Medical Research, Monash University, Vic. 3800, Australia*
[b] *Department of Biochemistry, Monash University, Vic. 3800, Australia*

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Abstract

Activin A and its binding protein, follistatin, are released into the circulation following acute systemic inflammation. In this study, we determined the activin and follistatin response of ovine aortic endothelial cells to lipopolysaccharide (LPS). Exposure to LPS for 1 h, mimicking a transient inflammatory event, elicited significant increases in activin β_A subunit mRNA or activin A release, with larger, more prolonged increases evident with continuous exposure. On the other hand, follistatin increases were only evident with prolonged exposure to LPS and following increases in activin A release. While cell-associated activin A increased with LPS exposure, levels were lower than those secreted, whereas the opposite was apparent for follistatin. In summary, our findings suggest that vascular endothelial cells, while capable of releasing activin A and follistatin following inflammatory stimulation, are unlikely to be responsible for the rapid release of activin A in vivo following inflammatory challenge.
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1. Introduction

Activins are members of the transforming growth factor-β (TGF-β) superfamily which bind to dimeric receptors and regulate a broad range of cellular functions (Chang et al., 2002; Phillips and de Kretser, 2003). Although five activin β subunit forms have been identified (β_A–β_E), the most well characterised is activin A, consisting of a homodimer of β_A subunits. The inhibins (consisting of a β subunit and a structurally distinct α-subunit), are also recognised for their roles in reproduction and development (de Kretser et al., 2004; Muttukrishna et al., 2004). An activin binding protein, follistatin, has also been identified and its biological significance lies in its ability to bind and neutralize activins with high affinity, in addition to other TGF-β family members (Phillips and de Kretser, 1998).

Specific changes in the expression or synthesis of activins in a variety of cell types suggest they are involved in a plethora of physiological and pathological processes including inflammation (Phillips et al., 2001), where activin A has been purported to be potentially pro-inflammatory (Shao et al., 1998) or anti-inflammatory (Yu and Dolter, 1997; Ohguchi et al., 1998; Yu et al., 1998) depending upon the end-points examined and experimental system used. Circulating levels of activin A and follistatin are elevated in clinical sepsis (Michel et al., 2003), a systemic inflammatory condition. Our understanding of the factors which determine the development and aetiology of sepsis is limited, particularly which mediators and factors are critical in the progression of the condition. Our group has developed an ovine sepsis model to mimic the effects of systemic bacterial infection. We have found that injection of the bacterial cell wall component, lipopolysaccharide (LPS), elicits a rapid release of a range of biologically active mediators, with activin A among the first of the cytokines to be appear in the circulation. The role activin may play in systemic inflammatory events is currently being examined and a second avenue of investigation has been to ascertain the source of the activin A, which rises 10-fold over basal levels within 50 min of LPS injection (Jones et al., 2000, 2004).

Both monocytes and macrophage are known to produce activin A in response to prolonged LPS exposure in vitro (Shao et al., 1992). In addition, it has been reported that porcine aortic endothelial cells (which express the genes for both the activin β_A subunit and follistatin) demonstrate elevated expression of follistatin upon exposure to LPS after as little as 4 h (Michel et al., 1996). Certainly, vascular endothelial cells are abundant in

* Corresponding author. Tel.: +61 3 9594 7002; fax: +61 3 9594 7114.
E-mail address: david.phillips@med.monash.edu.au (D.J. Phillips).

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vivo and represent a cell type which would be directly exposed to circulating LPS. For these reasons we hypothesised that, in our in vivo model of sepsis and in clinical manifestations of this disease, endothelial cells may contribute to the rapid rise in circulating activin A, as well as the later observed elevation of follistatin.

To examine this possibility, we have taken an in vitro approach to characterise the capacity of ovine aortic endothelial cells (OAEC) to synthesise and release activin and follistatin in response to LPS exposure. In the ovine sepsis model previously described by our group (Jones et al., 2000, 2004), we estimate plasma concentrations of injected LPS would reach 1–10 ng/ml, before undergoing rapid pulmonary clearance (Warner et al., 1988). Consequently, this model may best reflect the systemic processes involved in acute sepsis, rather than those associated with chronic sepsis, where the endothelium may be exposed to LPS for extended periods of time. Nevertheless, in the studies we report here we have used in vitro strategies to mimic both these situations. We examine the effects of both transient and sustained LPS exposure upon endothelial cells for up to 24 h. Our studies also assess the cellular and released levels of activin A and follistatin and how, over time, LPS affects the synthesis, storage and release of these mediators.

2. Materials and methods

2.1. Culture of endothelial cells

OAEC were originally obtained from aortae of young sheep (Norman et al., 1999) and cultured as described for bovine cells (Lew et al., 1994). Over a number of passages, they displayed typical staining for the endothelial cell marker, von Willebrand factor (data not shown). Cellular morphology was typical for endothelial cells, with monolayered 'cobblestone' morphology and the absence of contaminating fibroblasts or pericytes. Cells were cultured on a 0.2% gelatin substrate and passaged using 0.1% trypsin–0.02% ethylenediaminetetra–acetic acid (EDTA). They were cultured in RPMI 1640 medium (Gibco, Invitrogen Corp., Auckland, New Zealand) supplemented with 25 U/ml penicillin and 25 μg/ml streptomycin, L-glutamine (1 mM), non-essential amino acids and 10% heat inactivated foetal calf serum (Gibco). Cells at passage numbers six to nine were seeded into 0.2% gelatinised 12-well plates and following one medium change, were used at confluence ($>2.0 \times 10^4/cm^2$) 48 h after seeding. LPS (*E. coli* serotype 0127:B8, Sigma Chemical Company, St. Louis, MO, USA) was diluted in culture medium and cells were exposed to final concentrations of 1–1000 ng/ml for between 1 and 24 h. Treatments were undertaken in duplicate and results are from two to five independent experiments.

2.2. Immunoassays

Activin A and follistatin concentrations were determined by previously validated immunoassays that measure 'total' amounts, that is, both 'bound' and 'free' components (Knight et al., 1996; O'Connor et al., 1999). The release of activin A and follistatin from cells was determined in cell supernatants and concentrations were typically described as ng/10^6 cells. Cell counts were performed by removing the supernatant and resuspending cells using trypsin-EDTA. This cell suspension was also used, following cell lysis, to evaluate cell-associated activin A and follistatin concentrations. Both analytical assays included appropriate controls to correct for any endogenous activin or follistatin levels in samples. Minimal quantifiable concentrations were, for activin A, 0.01 ng/ml and follistatin, 2.3 ng/ml. The intra- and inter-assay coefficients of variation over 16 separate assays were 6.8% and 8.8% and both <12% for the activin A and follistatin assays, respectively.

2.3. Gene expression studies

For analyses of activin β_A subunit and follistatin gene expression levels, RNA was extracted from cell monolayers using Trizol (Invitrogen Corp., Carlsbad, CA, USA) in accordance with the manufacturer's specifications. RNA was then treated with DNAse I (Ambion Inc., Austin, TX, USA) to eliminate genomic DNA contamination. cDNA was derived from OAEC exposed to LPS to give high expression levels of the genes of interest using Super Script II or III Rnase H^- Reverse Transcriptase (Invitrogen Corp.). Quantitative real time PCR was performed using a Roche LightCycler (with Light Cycler-DNA Master SYBR Green I; Roche Diagnostics GmbH, Penzberg, Germany). This allowed the determination of activin β_A subunit and follistatin expression relative to that of β-actin (the expression of which was not affected by the treatments used). Expression data were then represented as fold change in expression, relative to untreated, time-matched controls.

Primers used in the real time PCR analyses were designed based on published gene sequences using Primer 3 software. Annealing temperatures used for all were 60 °C. PCR product length in base pairs (bp) is indicated and all PCR products were isolated, sequenced and assessed against published ovine sequences using NCBI Blast to confirm they represented products from the genes of interest. For β-actin, the primers were F = ccaaccgtgagaagatgacc (20 bp), R = caggactccatacccaggaa (20 bp), product = 475 bp; for follistatin, F = caacacgctcttcaagtgga (20 bp), R = gccaaccttgaagtcccata (20 bp), product = 574 bp; for the activin β_A subunit, F = gaagggaagaagagggatgg (20 bp), R = gacatgggtctcagcttggt (20 bp), product = 395 bp; for the inhibin α-subunit, F = agccgccctcaatatctctt (20 bp), R = ggttgggcaccatctcatac (20 bp), product = 281 bp.

2.4. Experimental details

The effects of continuous LPS exposure (1 ng/ml to 1 μg/ml) on activin A and FS release were first assessed over a period of 24 h. Time-dependent LPS-induced toxicity was evident above 1 ng/ml. Therefore, to reduce the confounding effects of cell toxicity upon assessments of activin and follistatin expression and synthesis, subsequent experiments were conducted using 1 ng/ml LPS.

The transient versus continuous exposure to LPS on released or cell-associated activin A and follistatin and associated gene expression levels was evaluated following a 5 min, 1 h or continuous LPS (1 ng/ml) exposure. Controls were included to evaluate activin and follistatin accumulation and gene expression and how the transient exposure protocol (which included a medium change) affected these end-points. LPS-induced changes were calculated relative to their time-matched, untreated controls and are represented as increases or decreases from these. All activin or follistatin data were standardised to cell counts. Replicates within experiments were averaged and results are represented as mean ± S.E.M. values from three independent experiments. Expression data were compared to the untreated 'continuous' control, in which expression levels were designated a value of 1.

2.5. Statistical analyses

Non-parametric Kruskall–Wallis analyses of grouped data were performed to ascertain factors contributing significantly ($P < 0.05$) to variance. Specific unpaired comparisons were made using Mann–Whitney *U*-test.

3. Results

3.1. Time- and concentration-dependent changes in OAEC viability to LPS

The effect on numbers of OAEC was established using graded doses of LPS for varying periods of exposure. Cell counts (representing adherent OAEC) in control wells remained consistent over the 24 h experimental period (Fig. 1). OAEC exposure to 1 ng/ml LPS produced no significant ($P = 0.11$) cell loss over

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