ACTIVIN A: EXPRESSION AND POTENTIAL SOURCES IN CARDIOPULMONARY BYPASS AND MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

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Submitted 2013

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ABSTRACT

Activin A, a member of the transforming growth factor-β superfamily of regulatory proteins, has been implicated in both acute and chronic inflammation. Neutralising its biological effects using follistatin, a naturally occurring high affinity binding protein, has been associated with improved outcomes in animal models of inflammatory conditions such as endotoxaemia, inflammatory bowel disease, asthma and renal ischaemia-reperfusion injury. Cardiac surgery is associated with a generalised non-specific inflammatory response and this is largely an effect of cardiopulmonary bypass and myocardial ischaemia-reperfusion injury. In this thesis, the release pattern of activin A and follistatin in the setting of cardiopulmonary bypass and myocardial ischaemia-reperfusion was studied. Cardiopulmonary bypass was associated with a biphasic pattern of activin A release. Follistatin was also robustly released by cardiopulmonary bypass and the serum levels correlated with serum interleukin-6 levels. Activin A expression was increased in myocardial ischaemia-reperfusion and pre-treatment with follistatin reduced infarct size associated with ischaemia-reperfusion. Lastly, neutrophils were identified as a potential source of preformed activin A in acute inflammation. Tumour necrosis factor-α was able to stimulate the release of activin A from neutrophils via a p38 MAP kinase dependent pathway. Overall, this thesis has provided data to support the hypothesis that the activin A/follistatin axis is involved in the inflammatory response to cardiopulmonary bypass and myocardial ischaemia-reperfusion. Circulating neutrophils may be a potential source of preformed activin A in the setting of acute inflammation. Follistatin may have therapeutic potential in ameliorating myocardial ischaemia-reperfusion injury.

GENERAL DECLARATION

Monash University

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals and one submitted for publication. The core theme of the thesis is regulation and effects of activin A and follistatin in cardiopulmonary bypass and ischaemia reperfusion related inflammation. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Surgery (MMC) and Centre for Reproductive Biology, Monash Institute of Medical Research under the supervision of Dr David J Phillips, A/Prof Mark P Hedger and Prof Julian A Smith.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chpaters 2, 3 and 4, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Pattern of activin a and follistatin release in a sheep model of cardiopulmonary bypass	Published	Design of experiments, laboratory work, data analysis and preparation of manuscript; 65%
3	Regulation and actions of activin A and follistatin in myocardial ischaemia-reperfusion injury	Submitted	Design of experiments, laboratory work, data analysis and preparation of manuscript; 65%
4	Tumour necrosis factor-α stimulates human neutrophils to release preformed activin a	Published	Design of experiments, laboratory work, data analysis and preparation of manuscript; 65%

I have / have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted]

Date: **3/3/2013**

ACKNOWLEDGEMENTS

First I would like to thank my supervisors Dr David Phillips, A/Prof Mark Hedger and Prof Julian Smith for all their time and effort over the last 5 years. I could not have done this without their patient and expert guidance. I would also like to thank Sue Hayward, Hui Wu, Wendy Winnall, Mei Ling Lim, Penny Clay, Catherine Itman, Craig Harrison, Jim McMillan, Nikolai Faizoulline and Jacob Goldstein for all their help along the way. Finally a very big thankyou to my mum, to whom I owe everything that I have achieved.

PRESENTATIONS AND PUBLICATIONS

Oral presentations

1. Chen Y, Phillips DJ, McMillan J, Bedford P, Goldstein J, Wu H, Hedger MP and Smith JA. Pattern of activin A release in a sheep model of CPB – preliminary results. *Australian Society of Cardiac and Thoracic Surgeons Annual Scientific Conference, Noosa 2008*
2. Chen Y, Rothnie C, Spring DJ, Verrier ED, Venardos K, Kaye D, Phillips DJ, Hedger MP and Smith JA. Follistatin, an activin A binding protein, reduces infarct size in myocardial ischaemia reperfusion injury. *Australian Society of Cardiac and Thoracic Surgeons Annual Scientific Conference, Noosa, Australia 2009* ***(TAG Medical Young Achievers Award)***
3. Chen Y, Wu H, Winnall WR, Loveland KL, Makanji Y, Phillips DJ, Smith JA and Hedger MP. Tumour necrosis factor stimulates human neutrophils to release preformed activin A. *The Endocrine Society of Australia 53rd Annual Scientific Meeting, Sydney 2010*

Journal publications

1. Chen Y, Phillips DJ, McMillan J, Bedford P, Goldstein J, Wu H, Hedger MP and Smith JA. Pattern of activin A and follistatin release in a sheep model of cardiopulmonary bypass. Cytokine 2011; 54(2):154-60
2. Chen Y, Wu H, Winnall WR, Loveland KL, Makanji Y, Phillips DJ, Smith JA and Hedger MP. Tumour necrosis factor-α stimulates human neutrophils to release preformed activin A. Immunology and Cell Biology 2011; 89(8):889-96
3. Wu H, Chen Y, Winnall WR, Phillips DJ and Hedger MP. Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 2012; 303(6):R665-75
4. Wu H, Chen Y, Winnall WR, Phillips DJ and Hedger MP. Regulation of activin A release from murine bone marrow-derived neutrophil precursors by tumour necrosis factor. Cytokine 2013; 61(1):199-204
5. Wu H, Wu M, Chen Y, Allan CA, Phillips DJ, Hedger MP. Correlation between blood activin levels and clinical parameters of type 2 diabetes. Experimental Diabetes Research (in press)

LIST OF ABBREVIATIONS

ACTR	Activin receptor
ALK	Activin receptor like kinase
ANF	Atrial natriuretic factor
ARDS	Acute respiratory distress syndrome
AUC	Area under the curve
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
CCF	Congestive cardiac failure
CNS	Central nervous system
CPB	Cardiopulmonary bypass
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DAMP	Danger associated molecular pattern
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FSH	Follicle stimulating hormone
FSRP	Follistatin related protein
FSTL3	Follistatin like 3
GDF	Growth differentiation factor
HSPG	Heparan sulphate-containing proteoglycan
HMGB1	High mobility group protein B1
HR	Hypoxia reoxygenation
HSP	Heat shock protein
INF	Interferon
IL	Interleukin
IR	Ischaemia reperfusion
JNK	c-Jun N-terminal kinase
LAD	Left anterior descending artery
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEKK	MAPK/ERK kinase kinase
MMP	Matrix metalloproteinase
MOD	Multiple organ dysfunction
MPTP	Mitochondrial permeability transition pores
NFκB	Nuclear factor kappa B
NLRP3	Nod-like receptor family, pyrin domain containing 3
NO	Nitric oxide
NOS	Nitric oxide synthase
NVCM	Neonatal ventricular cardiomyocyte
PACE	Paired basic amino acid cleaving enzyme
PAMP	Pathogen associated molecular pattern

RAGE	Receptor for advanced glycation endproducts
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
TAK	TGF-β activated kinase
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TRAF	TNF receptor associated factor
TRIF	TIR domain-containing adapter inducing interferon-beta

CHAPTER 1

LITERATURE REVIEW

1. Activin A and follistatin

Activin A, a member of the TGF-β superfamily of growth regulatory proteins, was originally described for its role in stimulating the release of FSH from the pituitary gland (Ling et al. 1986). It has since been shown to play a more diverse role in the regulation of inflammation, immunity and cell proliferation (Phillips et al. 2009). Follistatin binds activin A with high affinity and is an endogenous negative regulator of its bioactivity. This section summarises the current understanding of activin and follistatin biology.

1.1 TGF-β superfamily classification and structure

There are over 40 members in the TGF-β superfamily. These can be divided into subfamilies according to structural and functional similarities. These are: 1) TGF-β subfamily (TGF-β1-3); 2) inhibins (A, B and others) and activins (A, B and others) subfamily; 3) BMPs and GDFs, and 4) other divergent members, such as anti-Müllerian hormone and Nodal. The amino acid sequence of translated prepropeptide of the TGF-β superfamily members consists of an N-terminal signal peptide, a pro-domain and a mature domain. The signal peptide targets the peptide for uptake by the rough endoplasmic reticulum and the Golgi apparatus for further processing. The pro-domain is enzymatically cleaved from the mature domain. Although there are significant variations in the pro-domain amino acid sequence amongst the superfamily members, the region is thought to be important for the correct folding and the secretion of the mature protein (Kingsley 1994). In the case of TGF-β, the pro-domain is non-covalently linked to the mature domain and both are secreted as an inactive latent complex requiring proteolysis (e.g. plasmin or thrombospondin) *in vivo* or lowering of pH, boiling or SDS treatment *in vitro* for activation (Miyazono 2008). The association between the pro-domain and the mature peptide in other members of the superfamily, such as the activins and BMPs, is significantly weaker and the pro-domain is readily displaced upon receptor binding and therefore these members

are secreted in an active form lacking the pro-domain. The mature protein is characterised by a highly conserved six to nine cysteine residue sequence in the mature domain (cystine knot scaffold) which form intra- and intermolecular disulphide bonds. These proteins are typically secreted as homo- or hetero-dimers of the mature subunits, adopting a butterfly shaped configuration linked by disulphide bonds **(Figure 1)**.

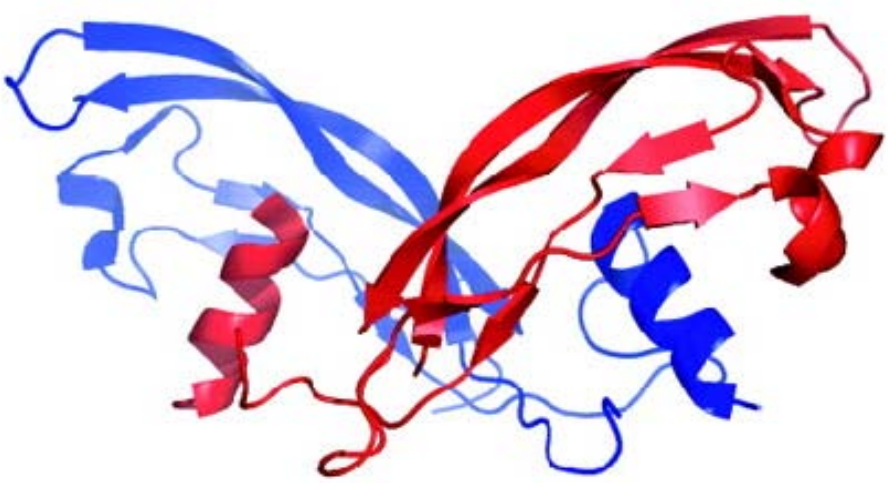


Figure 1 *Activin structure: homodimers of two inhibin β subunits (red and blue) adopting a butterfly configuration, linked by disulphide bonds. From (Lin et al. 2006b)*

1.2 Activins

Activins belong to the activin/inhibin subfamily. While inhibins are disulphide linked heterodimers of inhibin α and β subunits, activins are homodimers of the inhibin β subunits only (Lin et al. 2006b). Four β-subunit genes (A, B, C and E) have been described in human (β_D having been described in Xenopus but not humans). Of these, only activin A (two β_A), activin B (two β_B) and activin AB (β_A and β_B) have been found to be biologically active (Thompson et al. 2004). Little is known about the function of the other activins.

Activin A and activin B were originally isolated from porcine follicular fluid during purification of inhibin (Ling et al. 1986). Activin A is by far the best characterised of the activin group. Activin A releases FSH from the pituitary gland, whereas inhibin suppresses the release of FSH by antagonising activin A (Gregory and Kaiser 2004). In addition to its role in reproduction, activin A has also been identified as an erythroid and megakaryocyte differentiation factor (Broxmeyer et al. 1988; Eto et al. 1987), neuronal survival factor (Schubert et al. 1990), mesoderm-inducing factor (Smith et al. 1990) and pro-apoptotic factor (Nishihara et al. 1993; Schwall et al. 1993). More recently, there is an increasing appreciation of its role in inflammation, fibrosis and immune regulation (Hedger et al. 2011).

The human βA gene is localised to Chromosome 7 (7p15-p13). The mRNA produces a precursor protein of 425 amino acids with a molecular mass of 59 kDa. As with other members of the TGF-β superfamily, the β_A monomer is synthesised *in vivo* as a pre-pro-peptide. The proteolytic cleavage of the propeptide is mediated by furin, also known as PACE, a member of the subtilisin-like proprotein convertase family. It cleaves the propeptide at the highly conserved dibasic amino acid cleavage site (R-X-X-R), removing the pro-domain to release the mature C-terminal form of 116 amino acids (13kDa). The mature peptide forms an inter-subunit disulphide bond via Cys79 and is secreted as a homodimer. Structurally it is homologous to other members with a highly conserved cysteine knot scaffold and a butterfly configuration.

1.3 Activin A receptors and intracellular signalling

Receptors of the TGF-β superfamily belong to the serine/threonine kinase family of receptor kinases. Activin A binds to the type II receptors which are constitutively active and they in turn recruit and phosphorylate type I receptors. There are seven type I and five type II receptors (Shi and Massague 2003). Activin A binds to type II receptors (ACTRII), which in turn recruit an activin type 1 receptor, ACTRIB (also

known as ALK4) (Feng and Derynck 2005). This pattern of sequential receptor recruitment applies to both the TGF-βs and activins. In contrast, BMPs and GDFs are able to bind to either receptors but signalling only occurs when both receptors are bound.

The canonical intracellular signalling pathway for activin A is via the SMADs, a group of mammalian signal transduction proteins homologous to the Drosophila protein, MAD and the *Caenorhabditis elegans* protein, SMA (Wrana and Attisano 2000). The system of SMADs is activated by the type I receptors. The SMAD proteins are classified according to their functions. There are receptor regulated SMADs, SMAD1, SMAD5 and SMAD8/9 (activated by ALK1, ALK2, ALK3 and ALK6 type 1 receptors) and SMAD 2 and SMAD3 (activated by ALK4, ALK5 and ALK7 type 1 receptors), the common SMAD (SMAD4) and inhibitory SMADs, SMAD6 and SMAD7. Activin A binds to ALK4 and therefore signals via SMAD2 and SMAD3. Following phosphorylation, these SMADs complex with SMAD4 and are transported into the nucleus and activate specific target genes.

There is also evidence that TGF-β and activin A may signal through MAPK to activate the p38/JNK pathways (Derynck and Zhang 2003). This action is independent of the SMAD signalling and does not require type I receptor kinase activity. It appears to signal via TRAF6 and the recruitment of TAK-1 (Sorrentino et al. 2008; Yamashita et al. 2008). This pathway is utilised by activin A to mediate the growth inhibition effect on human breast cancer T47D cells in addition to SMAD signalling (Cocolakis et al. 2001). In addition, activin A's effect on keratinocyte migration is mediated by MEKK1-dependent JNK and p38 activation, but not on SMAD signalling (Zhang et al. 2005).

1.4 Activin A binding proteins and antagonists

There are a number of structurally unrelated proteins that bind to activin A and neutralise its biological activity. These include follistatin and FSRP, also known as FSTL3. A number of other proteins (Crypto and BAMBI) have been found to have the ability to inhibit activin signalling via interaction with the activin-receptor complex, but their biological significance in the regulation of activin A signalling is still unclear (Gray et al. 2003; Onichtchouk et al. 1999).

1.4.1 Follistatin

Follistatin contains a 63-residue N-terminal segment followed by three follistatin domains (FS1-3) each of 73-75 amino acids. These domains each contain 10 cysteine residues. Two main forms of follistatin exist by alternative splicing, FS288 and FS315. FS288 lacks the last exon due to inclusion of an intronic sequence that contains an early stop codon (Shimasaki et al. 1988). FS288 resides mainly in the tissue as it binds cell surface HSPGs with high affinity (via its FS-1 domain). On the other hand, FS315 is considered to be the predominant circulating form of follistatin, because it only binds to heparan sulphate after forming a complex with activin A which alters its conformation and exposes the HSPG binding sites. It is speculated that FS315 binds free circulating activin A, the complex then in turn binds to cell surface HSPGs for internalisation and degradation. A systemically administered anticoagulant, heparin, has been shown to increase circulating activin A and follistatin levels concurrently. This is thought to be due to the structurally analogous heparin displacing cell surface HSPG-bound activin and follistatin complexes (Jones et al. 2004b).

Follistatin binds to activin A with high affinity (50-500pM). Activin A binds to the N-terminal 63 residue region, as well as the FS-1 and FS-2 domains and these are essential for suppressing its biological effects (Thompson et al. 2005). Two follistatin

molecules bind one activin A dimer and this obstructs each activin subunit's type II receptor binding site. Follistatin does not bind directly to activin receptors. Its only known biological function is through its binding of TGF-β superfamily members. Follistatin binds other members of the TGF-β superfamily at a lower affinity than activin A binding. For example, follistatin can also bind to activin B but the affinity for activin B is 10 times lower than for activin A (Schneyer et al. 2003). It can also bind with lower affinity to myostatin (GDF8), GDF9 and BMPs 6 and 7, but not TGF-β1 or TGF–β2 (Sidis et al. 2006) (Hedger et al. 2011). Binding of follistatin to TGF-β3 has been described to inhibit epithelial-mesenchymal transition *in vitro* (Nogai et al. 2008).

1.4.2 FSRP

FSRP also binds to activin A with high affinity (850pM) (Harrison et al. 2005). It differs from follistatin in lacking the FS-3 domain and the consensus heparin-binding sequence (Schneyer et al. 2001). Therefore it cannot bind to cell surface HSPGs. The tissue distribution pattern of FSRP is also different from that of follistatin. It is more highly expressed in the placenta, the testis, the skin and the cardiovascular tissues, whereas follistatin expression is highest in the pituitary, the ovaries and the male reproductive organs (Schneyer et al. 2001). Although a number of other follistatin-like domain containing proteins have been described, they do not seem to bind activin A (Geng et al. 2011; Phillips and de Kretser 1998).

2. Activin A and acute inflammation

Activin's role in the immune system and inflammation was first suggested through its ability to regulate thymocyte proliferation (Hedger et al. 1989). Subsequently, monocytes and bone marrow stromal cells were found to produce functional dimeric activin A following stimulation by LPS and cytokines such as IL-1α and TNF-α (Shao et al. 1992). Following this, a series of papers have shown that activin A production is stimulated by inflammatory mediators in a variety of cell types and that it, in turn, is able to induce the production of pro-inflammatory cytokines from immune and stromal cells. Levels of activin A in serum are elevated in a large number of clinical conditions in which inflammation is known to be of aetiological importance. These conditions include liver cirrhosis, chronic renal failure, advanced solid tumour cancer (Harada et al. 1996), congestive heart failure (Yndestad et al. 2004), pulmonary hypertension (Yndestad et al. 2009b) and acute myocardial infarction (Miyoshi et al. 2009). This section will focus on the inflammatory conditions associated with elevated serum and tissue activin A levels and will also examine the function of activin A in the setting of inflammation.

2.1 Activin A release in animal models of systemic inflammation

The study of activin A in systemic inflammation was instigated by the finding that follistatin is released systemically in response to surgical trauma or IL-1β infusion (Phillips et al. 1996). Elevated follistatin serum levels were also demonstrated in patients with septicaemia (Michel et al. 1998). These observations led to the study of activin A levels in animal models of LPS-induced inflammation. LPS signals via the TLR4 receptor and triggers a whole body inflammatory response characterised by raised body core temperature, release of pro-inflammatory cytokines and hemodynamic disturbances (endotoxaemia). Following systemic administration of LPS, circulating activin A levels increase rapidly and in a biphasic pattern (Jones et al.

2000; Jones et al. 2007). The first serum peak occurs less than one hour following LPS challenge, while a smaller and less defined second peak occurs 4-12 hours later. Serum follistatin level is also increased, with its peak largely coinciding with the second peak of the activin A response. The first peak in activin A response coincides or is slightly earlier than the systemic release of TNF-α, which is one of the earliest cytokines to be released in endotoxaemia (Mant et al. 2008; Van Zee et al. 1995), and precedes the release of IL-1β and IL-6. This pattern of activin A release to LPS has been demonstrated to be reproducible in both sheep and mice (Jones et al. 2000; Jones et al. 2007) **(Figure 2)**. These studies demonstrated for the first time that activin A is a critical early cytokine that is released in LPS-induced systemic inflammation. Although the release of activin A in response to LPS has not been demonstrated in humans, a number of studies suggest that both activin A and follistatin levels are increased in bacterial sepsis (see below 2.2.1)

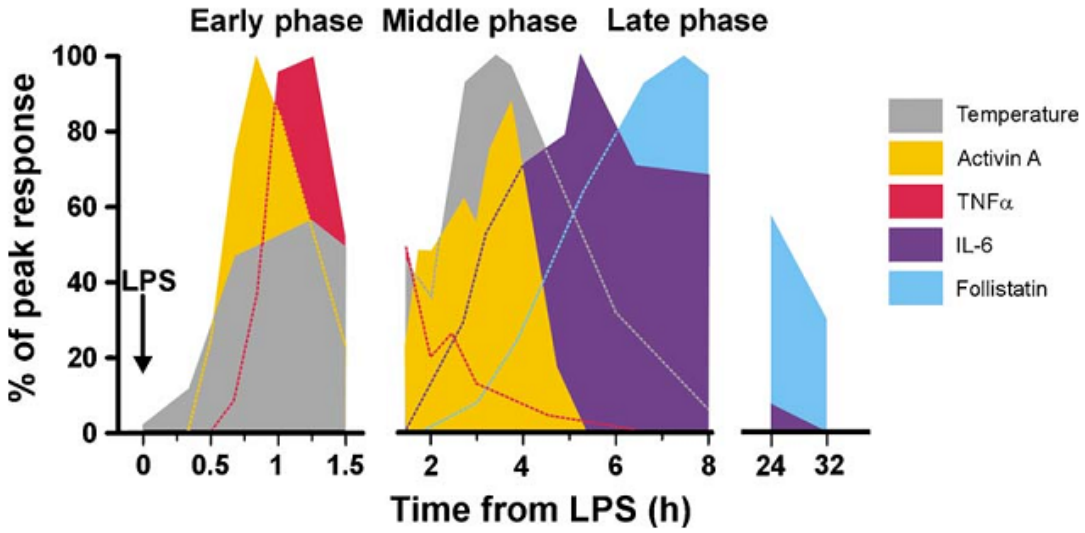


Figure 2 *Release profile of inflammatory cytokines following LPS administration and changes in core body temperature in a sheep model of acute inflammation. From (Phillips et al. 2009)*

2.2 Activin A release in infective and sterile inflammatory conditions

In addition to animal models of LPS-induced systemic inflammation, a large number of *in vivo* and *in vitro* studies have examined the release and the function of activin A in the setting of infective and sterile inflammation. The ability of follistatin to neutralise the adverse effects of activin A in these conditions has also been explored.

2.2.1 Infection

Michel and colleagues have demonstrated elevated activin A and follistatin levels in patients presenting with septicaemia. The levels correlated with serum CRP levels, however no statistically significant correlation with clinical outcomes was found (Michel et al. 2003a). Neonates with hospital-acquired infections also have elevated serum activin A levels (Petrakou et al. 2008). Activin A levels in the CSF are significantly increased following bacterial meningitis (Ebert et al. 2006; Michel et al. 2003b). This occurs without changes in serum activin A levels and is thought to be produced by microglial cells and infiltrating macrophages in response to bacterial components in the CSF (Michel et al. 2003b). In another study, Wilms and colleagues were able to show that activin A levels were elevated in bacterial but not viral meningitis (Wilms et al. 2010). Although the functional significance of elevated activin A in infection has not been fully elucidated, these studies clearly suggest that infection is a trigger for the systemic release of activin A and follistatin.

2.2.2 Trauma

Infection is not the only condition in which activin A and follistatin levels are elevated. The same innate immune pathways are activated in sterile inflammation. Not surprisingly, activin A and follistatin levels have been studied in this setting. In a study by Phillips and colleagues, CSF activin A level was found elevated in a subpopulation of traumatic brain injury, in which the activin A level correlated with CSF levels of neuronal specific enolase and S100-β, both of which are markers of

cell necrosis (Phillips et al. 2006). S100 proteins signal through RAGE and activate pro-inflammatory pathways including NFκB and MAPK, both of which are possible regulators of the activin A secretion pathway (Hofmann et al. 1999). There has not been any study that specifically examined whether surgical trauma itself causes release of activin A. However, an early study done before an activin A immunoassay was readily available found increased levels of follistatin following sham castration, an effect thought to be due to surgical trauma alone (Phillips et al. 1996). These studies do not elucidate the mechanism of activin A increase in the setting of trauma. However, it is increasingly appreciated that tissue damage is associated with the release of endogenous molecules that can elicit an inflammatory response and this may be the trigger for activin A release in this setting.

2.2.3 Inflammatory arthropathies

Activin A levels are elevated in synovial fluids in inflammatory arthropathies such as rheumatoid arthritis and gout, but not in degenerative osteoarthritis (Yu et al. 1998). Fibroblast-like synoviocytes and CD68-positive macrophages in rheumatoid synovium can secrete activin A when stimulated with IL-1β, TNF-α and TGF-β (Ota et al. 2003). It appears that activin is also a major downstream gene involved in IL-1Ra- and IL-10-mediated anti-inflammatory effects in rheumatoid arthritis, as double gene transfer of IL-1Ra and IL-10 alters the expression of activin A gene expression (Neumann et al. 2002). Urate crystal deposits within joints are the trigger for the inflammatory response in gout and these crystal deposits can be sensed by the NLRP3 inflammasome leading to the production of IL-1β, which is a potent stimulator for the synthesis and secretion of activin A (Martinon et al. 2006) (See section 2.3).

2.2.4 Inflammatory bowel disease

Inflammatory bowel disease is another condition in which the activin A expression is increased, where its mRNA levels correlate with expression of IL-1β and are found

highest in areas of greatest inflammation (Hubner et al. 1997). Activin A mRNA expression is predominantly localised in the mucosa and submucosa of inflamed intestine. In mouse models of inflammatory bowel disease, follistatin has been shown to reduce the severity of colonic inflammation and improve survival (Dohi et al. 2005).

2.2.5 Allergic inflammation

Activin also seems to be a target gene for ovalbumin-induced allergic airway inflammation, in which activin mRNA is significantly up-regulated without a concomitant increase in TGF-β expression (Rosendahl et al. 2001). Infiltrating mast cells have been identified as an importance source of activin A in this setting (Cho et al. 2003). CD4+ T cells from patients with asthma and atopic dermatitis have increased activin A expression (Wohlfahrt et al. 2003). Ovalbumin challenge is associated with a significant up-regulation of nuclear phosphorylated SMAD2 in bronchial epithelium and alveolar macrophages and the intranasal administration of follistatin reduces the number of lymphocytes expressing IL-4 and IL-5 in the regional lymph nodes (Hardy et al. 2006). In addition, activin promotes airway smooth muscle proliferation (Cho et al. 2003). IL-13, a cytokine involved in mediating type 2 immune response, increases bronchial epithelial activin A production (Hardy et al. 2010). Recently, it was also shown that eosinophils were able to produce activin A in response to a combination of IL-3 and TNF-α (Liu et al. 2010).

2.2.6 ARDS

In addition to allergic disorders of the lung, pulmonary overexpression of activin A has been shown to cause acute lung inflammation in mice similar to that seen in ARDS (Apostolou et al. 2012). ARDS is a clinicopathological entity with diverse inflammatory aetiologies including septicaemia and severe trauma. In patients with ARDS, the authors have also found there was a selective upregulation of activin A protein levels in the bronchoalveolar lavage fluids of these patients. The authors

further demonstrated that neutralisation of activin A activity led to attenuated lung pathology in a mouse model of ARDS.

2.2.7 Myocardial ischaemia

Yndestad and colleagues showed that serum activin A levels were elevated in patients with CCF and the level correlated with the degree of symptoms in these patients (Yndestad et al. 2004). Further, myocardial gene expression of both TGF-β and activin A is increased in the myocardium in CCF (Mahmoudabady et al. 2008). Activin is able to upregulate expression of cardiac remodelling genes such as ANF, MMP-9 and TGF-β in this setting. In other studies, serum activin A levels are elevated in patients with chronic stable angina pectoris and the levels of activin A prior to patients undergoing percutaneous coronary interventions are an independent predictor of the release of creatinine kinase following the procedure (Miyoshi et al. 2009; Smith et al. 2004). Interestingly, mRNA levels of activin A in peripheral T cells in patients with unstable angina is less than in patients with stable angina (Smith et al. 2004). Unstable angina represents a progression from stable angina due to inflammation and the authors speculated that activin A might have an anti-inflammatory role in this aspect.

Not only is activin A involved in the chronic remodelling process of the heart, it has also been shown recently to be elevated secondary to myocardial IR injury. In a rat myocardial IR model, following 30 minutes of ischaemia and 60 minutes of reperfusion, myocardial activin A levels were significantly increased compared with surgical shams, and post-conditioning of the heart (a mechanism to make the tissue more resistant to IR) was able to decrease the expression of activin A following IR (Zhang et al. 2009a). In another study, activin A was found to be protective in myocardial IR and this was found to be due to its anti-apoptotic effect (Oshima et al.

2009). In addition to the heart, increased expression of activin A has also been found in the brain and the kidney following IR (Maeshima et al. 2001; Mukerji et al. 2007).

2.2.8 Malignancy

Cancer-related inflammation is another classical example of sterile inflammation. Many of the factors that stimulate inflammation are in play in the tumour microenvironment including hypoxia, cell necrosis and activated immune cells (Porta et al. 2011). An early study using a RIA to measure serum activin A levels had identified elevated levels in advanced solid cancers (Harada et al. 1996). In our laboratory, using a highly sensitive activin ELISA it has been found that activin A levels were significantly higher in metastatic cancers than either benign tumours or normal controls (unpublished data). Wildi and colleagues have shown elevated activin A mRNA in metastatic colon cancer (Wildi et al. 2001). Activin A levels are also elevated in breast cancer and interestingly, a decline in serum activin A level is seen in patients undergoing surgery for removal of breast cancer (Reis et al. 2002). A sterile inflammatory response is usually present in metastatic cancer and this may be the trigger for the chronically activated activin A level in this setting.

2.2.9 Burns

Activin is also highly expressed in injured skin. Activin A expression is normally found in fibroblasts in the dermis and the epidermis. Burns are associated with increased activin A level in the blister fluids and tissue infiltrate of immune cells that secrete activin A ((Phillips et al. 2009) and unpublished data from our laboratory).

These data suggest that the systemic release of activin A is not only confined to bacterial activation but also can be a result of tissue injury and inflammation. Activin A expression is highly inducible in sterile inflammation and a large number of *in vitro* studies have implicated the TLR pathway as well as pro-inflammatory cytokines such

as IL-1β and TNFα in the regulation of activin A synthesis and secretion. Although the endogenous stimuli for activin A expression in the individual conditions have not yet been fully elucidated, an overview of the regulation pathway will be briefly presented in Section 2.3.

2.3 TLR/IL-1 and the regulation of activin A synthesis and release

The TLRs belong to a group of receptors that have been collectively termed pattern recognition receptors (Chen and Nunez 2010). They play a fundamental role not only in the body's defence against microbial invasion but also in the cellular response to tissue injury and repair. They recognise conserved structural moieties that are found in microorganisms, commonly referred to as PAMPs. The TLRs function as dimers and recognize different microbial components. For example, TLR2 recognizes a number of bacterial and fungal wall components including lipoteichoic acid and beta-glucan. TLR4 recognizes LPS, a Gram negative bacterial wall component and TRL9 recognizes bacterial DNA motifs. Most TLRs signal via the MyD88 adaptor protein dependent pathway, except TLR3 which signals exclusively via the adapter protein, TRIF (Brikos and O'Neill 2008).

It is increasingly recognised that TLRs are not only microbial sensors but they also recognize endogenous molecules that are released as a result of tissue damage. Analogous to PAMPs, these endogenous molecules have been termed DAMPs. Molecules such as HMGB1, HSPs as well as extracellular matrix proteins (heparin sulphate, hyaluronan and biglycan) have been identified as endogenous activators of the TLR pathways (Chen and Nunez 2010). Elevated levels of activin A in conditions of sterile inflammation are therefore possibly mediated via the TLR pathways triggered by these endogenous DAMPs.

The animal endotoxaemia model studies suggest that activin A and its binding protein follistatin are important mediators of inflammation associated with microbial infection. Activation of the TLR4 by LPS triggers a robust and reproducible activin response *in vivo*. TLR4 mediated activin A release is dependent on the MyD88 adaptor protein as the response is completely abolished in MyD88 gene knockout (-/-) mice (Jones et al. 2007). Activin A release can also be triggered by agonists to TLR2 and TLR9, likely through MyD88 dependent pathway as well. The level secreted, however, is significantly lower than that induced by the TLR4 pathway ((Ebert et al. 2007) and unpublished data from our laboratory).

IL-1β is another key mediator in the sterile inflammatory response which also signals via the MyD88 dependent pathway. It is involved in the acute phase reaction, up-regulates adhesion molecule synthesis, and activates T cells and monocyte/macrophages to initiate cell-mediated immune responses. Like the TLRs, it has been shown to stimulate the synthesis and release of activin A in a large number of cell systems *in vitro* (Hedger et al. 2011).

The downstream signalling events that lead to activin A synthesis and release have not been fully elucidated but is likely to involve NFκB and AP-1 transcription factors, both of which are activated downstream of MyD88-dependent TLR and IL-1 signalling. NFkB is activated by phosphorylation and degradation of its IkB subunit while AP-1 can be activated by phosphorylation of the MAPKs, p38 and JNK (Hedger et al. 2011). Inhibition of NFκB and MAPKs reduce activin A expression *in vitro* in a number of culture systems (Hedger et al. 2011). Thus, there is a considerable body of *in vitro* data to suggest that TLRs are important regulators of *de novo* activin A synthesis. However, these findings have not been corroborated with *in vivo* studies in clinical conditions. The involvement of TLR/IL-1 in the regulation of sterile

inflammation-induced activin A release in the conditions described above is still largely speculative and further studies are required.

2.4 Functions of activin A

Like other TGF-β superfamily members, activin A has a wide range of cellular functions. An immunoregulatory role for activin A in acute inflammation is emerging (Hedger et al. 2011). In addition, activin has an established role in tissue fibrosis (Werner and Alzheimer 2006). This provides a link between acute and chronic inflammation in which activin A could be thought to exert its immunoregulatory and profibrotic effects as a continuum.

2.4.1 Pro-inflammatory and immunoregulatory effects

TGF-β has an established immunosuppressive role in inflammation. TGF-β or SMAD3 knockout mice have an overt inflammatory phenotype (Kulkarni et al. 1993; Matzuk et al. 1995). Unlike TGF-β, when the βA subunit gene is knocked out, neonatal lethality results, but this is not associated with an overt inflammatory phenotype (Matzuk et al. 1995). Although activin A expression is clearly up-regulated in a variety of acute inflammatory conditions, its exact role in this setting is still unclear.

In vitro data suggest that activin A may be pro-inflammatory as it stimulates the release of IL-1β, TNF-α and NO from monocyte/macrophage cell lines (Yamashita et al. 1993). However, the pro-inflammatory effect of activin A *in vitro* may not be very pronounced and is overshadowed by agents such as LPS. In rat bone marrow derived macrophage, LPS is more potent than activin A in eliciting inflammatory response and activin A had no additive effect to LPS. Activin A releases higher level of TNF-α in presence of a tyrosine kinase inhibitor (Nusing and Barsig 1999). Furthermore, pretreatment of microglial cells with activin A for 24 hours decreases

LPS induced inflammatory response at mRNA level and no additive pro-inflammatory effect is seen when given together with LPS (Sugama et al. 2007). In human monocytes, IL-1β processing is reduced with activin A treatment (Ohguchi et al. 1998). In gestational tissues, activin A at low concentration is able to stimulate, but at high concentration, inhibit the release of IL-6 and IL-8 (Keelan et al. 2000).

Activin A suppresses IL-6 synthesis (via TAK-1/JNK pathway) and decreases IL-6 effects on the acute phase response. It also inhibits B cell proliferation and the secretion of acute phase proteins in HepG2 human hepatoma cells and IL-6 proliferative response in M1 myeloblasts *in vitro* (Brosh et al. 1995; Yu et al. 1998). It appears to antagonise both the effect of IL-6 and IL-11 *in vitro*, by interfering with its signalling pathways (Brosh et al. 1995). Activated SMAD 3/4 complex acts as a transcription inhibitor of IL-6 targeted genes (Zauberman et al. 2001). It was also shown that activin A in sub-nanogram concentrations can induce apoptosis in plasmacytoma cell lines (Sternberg et al. 1995). Furthermore activin A inhibited proliferation in T cells (Hedger et al. 1989). Activin A is also highly inducible in dendritic cells and has been found to attenuate CD40 ligand specific cytokine production and promote dendritic cell differentiation and antigen uptake (Robson et al. 2008; Scutera et al. 2008). It induces macrophages to express arginase-1 (M2 phenotype) while inhibiting IFN-γ-induced NOS production (Ogawa et al. 2006). It also suppresses natural killer cell functions and contributes to the development of regulatory T cells (Huber et al. 2009; Robson et al. 2009; Semitekolou et al. 2009).

2.4.2 Proliferation, apoptosis and fibrosis

While it appears that activin A may play an immunoregulatory role in acute inflammation, its effect on cellular proliferation, fibrosis and apoptosis predominate in the more chronic setting. This is consistent with elevated serum levels of activin A in many chronic fibrotic conditions. Activin stimulates proliferation of lung fibroblasts,

porcine thyroid cells, keratinocytes and spermatogonial cells but increases apoptosis in hepatocytes, B lineage cells, prostate cancer cells and others (Chen et al. 2002). Activin A appears to inhibit cellular proliferation of parenchymal cells but promotes the production of cells involved in fibrosis (Werner and Alzheimer 2006).

The effect of activin on hepatocyte proliferation and apoptosis has been well studied. Activin A is constitutively expressed in normal liver parenchymal cells (De Bleser et al. 1997) and has been shown to suppress liver DNA synthesis and cause hepatocyte apoptosis (Hully et al. 1994; Schwall et al. 1993). Further, activin A inhibits the proliferative effect of EGF on hepatocytes (Yasuda et al. 1993). EGF induces activin A expression within hepatocytes which acts in an autocrine fashion to limit the extent of proliferation. Using follistatin can significantly accelerate the proliferative response of hepatocytes to EGF. Portal administration of follistatin after partial hepatectomy significantly accelerates liver regeneration (Kogure et al. 1995).

In a carbon tetrachloride-induced liver injury model, activin A receptor expression is depressed at 24 hours, resulting in less activin signalling and possibly rendering the hepatocytes more responsive to mitogen stimuli (Date et al. 2000). In a more chronic model, activin A expression was found to be decreased in the initial 2-3 weeks and thereafter increased when the liver had become fibrotic; activin was localised predominantly to hepatocytes around the areas of fibrosis (Huang et al. 2001). Serum levels of activin A are increased in chronic viral hepatitis as well as acute liver failure of various aetiologies (Hughes and Evans 2003; Patella et al. 2001). Serum activin A levels are also elevated in non-alcholic fatty liver disease and the levels correlate with the degree of hepatic fibrosis (Yndestad et al. 2009a). This is consistent with an early anti-proliferative effect of activin A and a later pro-fibrotic effect. Matrix components, such as biglycan, versican and heparin sulphate, are actively produced in the process of cirrhosis (Hogemann et al. 1997; Szende et al.

1992). These matrix components are also capable of activating TLR receptors (Kim et al. 2009; Schaefer et al. 2005) and may be partly responsible for the elevated activin A levels in liver cirrhosis. A reduced serum follistatin to activin ratio, which signifies relatively more abundant free activin A, is found in patients with acute hepatic failure compared with patients with acute hepatitis without failure (Lin et al. 2006a).

While activin A has a largely inhibitory effect on hepatocytes, it increases smooth muscle proliferation. Smooth muscle of the vasculature is able to synthesise activin A *in vitro* when stimulated with mitogens such as angiotensin II and thrombin (Pawlowski et al. 1997). It was found that activin A mediated the mitogenic effects of angiotensin II and thrombin in combination with EGF and heparin binding EGF-like growth factor, while these factors were only mildly mitogenic on their own. Activin A is also expressed in the smooth muscle of the pulmonary vasculature (Yndestad et al. 2009b). Serum activin A levels are increased in patients with pulmonary hypertension and the levels correlate with mortality (Yndestad et al. 2009b). Furthermore, activin A levels are markedly elevated in fibrotic lung conditions, such as a bleomycin-induced mouse lung fibrosis model and clinical conditions such as idiopathic pulmonary fibrosis and diffuse alveolar damage (Matsuse et al. 1995; Matsuse et al. 1996). The increased activin A expression is seen in the metaplastic epithelial cells, vascular smooth muscle, infiltrating macrophages and interstitial fibroblasts. Fibroblasts can both secrete and respond to activin A (Ohga et al. 1996; Ohga et al. 2000). They transform into myofibrolasts *in vitro* upon activin A treatment. Further, treatment with follistatin can ameliorate the pro-fibrotic effect of activin A on the lungs (Aoki et al. 2005).

Overall, it appears that the effect of activin A *in vivo* is more likely to be immunoregulatory than overtly pro-inflammatory in the acute phase of inflammation.

Activin promotes a type 2 response in activated macrophages and promotes antigen presentation by dendritic cells and antibody synthesis by B cells (Hedger et al. 2011; Ogawa and Funaba 2011) **(Figure 3)**. Type 2 immune responses have been linked to both pulmonary and hepatic fibrosis (de Jesus et al. 2004; Lukacs et al. 2001). Clinical and *in vitro* studies have consistently shown that activin A is involved in cellular proliferation and fibrotic processes. This suggests that activin A may be an orchestrator in the transition from acute inflammation to fibrosis.

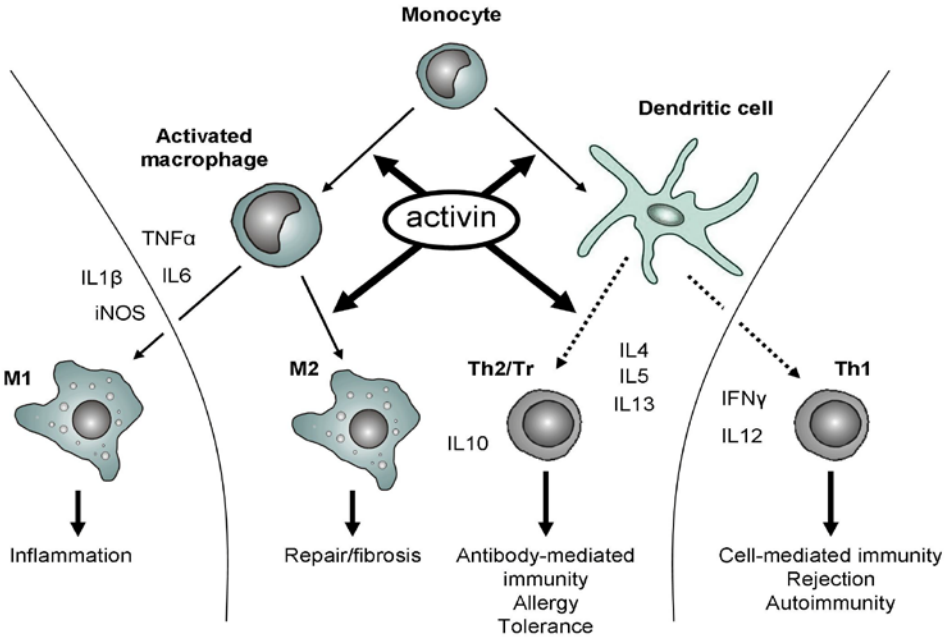


Figure 3 *Proposed immunological function of activin A. From (Hedger et al. 2011)*

2.5 Sources of activin A in acute inflammation

The βA subunit gene is widely expressed and virtually all cells in the body are able to produce activin A under appropriate stimuli. In the mouse embryo, activin A is expressed after day 10.5 in the mesenchymal cells of craniofacial regions, hair follicles, heart and digestive tract (Matzuk et al. 1995). In the human embryo, abundant levels of the βA subunit mRNA have been found in the heart, bone marrow and CNS (Tuuri et al. 1994). The βA subunit gene knockout is neonatally lethal, but

most defects seem to be limited to craniofacial skeletal development, with cleft palate resulting in a failure to suckle and death within 24 hours of birth (Matzuk et al. 1995). Thus, it appears that the lack of activin A does not impact pivotally on the development of most visceral organs.

The normal tissue distribution of activin A in adult humans has not been fully elucidated. However, mRNA expression levels in porcine tissues have been examined. βA subunit mRNA was found to have the highest levels in ovary, vasculature (vein and artery), uterus, adrenal gland and fat (Schneider et al. 2000). Studies from our laboratory have shown high levels of βA subunit mRNA in the liver of the mouse but using a sensitive and quantitative RT-PCR technique, detectable activin A mRNA was found in all major organs (Wu et al. 2012). Activin A protein has been immunolocalised in the lung (bronchial epithelial cells, alveolar macrophages) (Matsuse et al. 1996) (Yndestad et al. 2009b), bladder (transitional epithelial cells, smooth muscles) (Ying and Zhang 1995), vasculature (endothelial cells, smooth muscle) (Ying and Zhang 1995), digestive tract (intestinal epithelial cells, smooth muscle) (Dohi et al. 2005), cardiomyocytes (Yndestad et al. 2004), brain (cortical neurons, choroid plexus epithelial cells) (Michel et al. 2003b) and bone marrow (stromal cells, cells of macrophage lineage) (Wada et al. 1996), skin (keratinocytes, fibroblasts and hair follicle) (McDowall et al. 2008), male reproductive tract (Sertoli cells in testis) and female reproductive tract (Wada et al. 1996).

As noted in section 2.1, LPS initiates the rapid release of activin A protein. Studies in our laboratory have shown that the first peak of activin A release is only partially inhibited by inhibitors of protein synthesis (Wu et al. 2012), suggesting the release is not entirely from *de novo* protein synthesis but also involves preformed activin A

stored in tissues. However, with the wide spread tissue distribution of activin A, the precise cell source of this preformed activin A has not yet been identified.

2.6 Therapeutic potential of follistatin in inflammation and fibrosis

As discussed previously, follistatin binds activin A with high affinity. In a number of animal models of inflammatory conditions such as inflammatory bowel syndrome, renal and hepatic IR, asthma and LPS-induced endotoxaemia, follistatin has been shown to improve outcome and survival (Dohi et al. 2005; Hardy et al. 2006; Jones et al. 2007; Kanamoto et al. 2011; Maeshima et al. 2001). Not only can follistatin antagonise some of the acute effects of activin A but also it has been shown to reduce fibrosis in a number of chronic inflammatory conditions, including lung and liver fibrosis (Aoki et al. 2005; Patella et al. 2006). In transgenic mice with genetic overexpression of follistatin, skin wounds heal with less scarring (Wankell et al. 2001). The use of follistatin as a therapeutic agent has thus wide clinical applications although, to date, no clinical study has been published.

3. Cardiac surgery and inflammation

The majority of the cardiac operations today are carried out with the use of the heart lung machine which takes over the function of the heart and lungs in oxygenating and pumping the blood around the body, or CPB. The CPB circuit consists of a venous cannula which collects de-oxygenated blood from the heart, an oxygenator, an arterial cannula and a pump to deliver oxygenated blood back into the aorta, the main blood vessel in the body **(Figure 4)**. Once CPB is established, the heart is no longer required to pump blood around the body and therefore it may be stopped to allow the surgeon to operate on a stationary and bloodless field. The heart's beating is stopped through the use of the cardioplegia solution, which is a high potassium containing solution which arrests the heart in diastole. The delivery of cardioplegia solution requires the aorta to be cross-clamped so that the cardioplegia solution is only delivered into the coronary arteries, the blood supply of the heart, as these arteries are the only vessels arising proximal to the aortic cross-clamp. As a result, the heart is arrested while the rest of the body is continuously being perfused by the heart lung machine distal to the aortic cross-clamp **(Figure 4)**.

3.1 Triggers of the inflammatory response in cardiac surgery

It is well known that cardiac surgery is associated with a non-specific, whole body, sterile inflammatory response. In fact, an inflammatory response is seen in all surgical operations due to tissue trauma. Tissue destruction as a result of surgery is necessarily associated with an inflammatory response that is essential for tissue repair and healing. The degree of inflammation is thought to be related to the severity of tissue injury. The inflammatory response is similar in characteristics to the response to microbial infection due to activation of shared pattern recognition receptors such as TLRs, RAGE and Nods in response to the release of DAMPs. The whole spectrum of the innate immune response, including the acute phase response

and systemic release of pro- and anti-inflammatory cytokines, is initiated by surgical trauma. Surgical trauma may contribute more to the elevated inflammatory cytokine levels such as IL-6 than cardiopulmonary bypass itself (Prondzinsky et al. 2005)

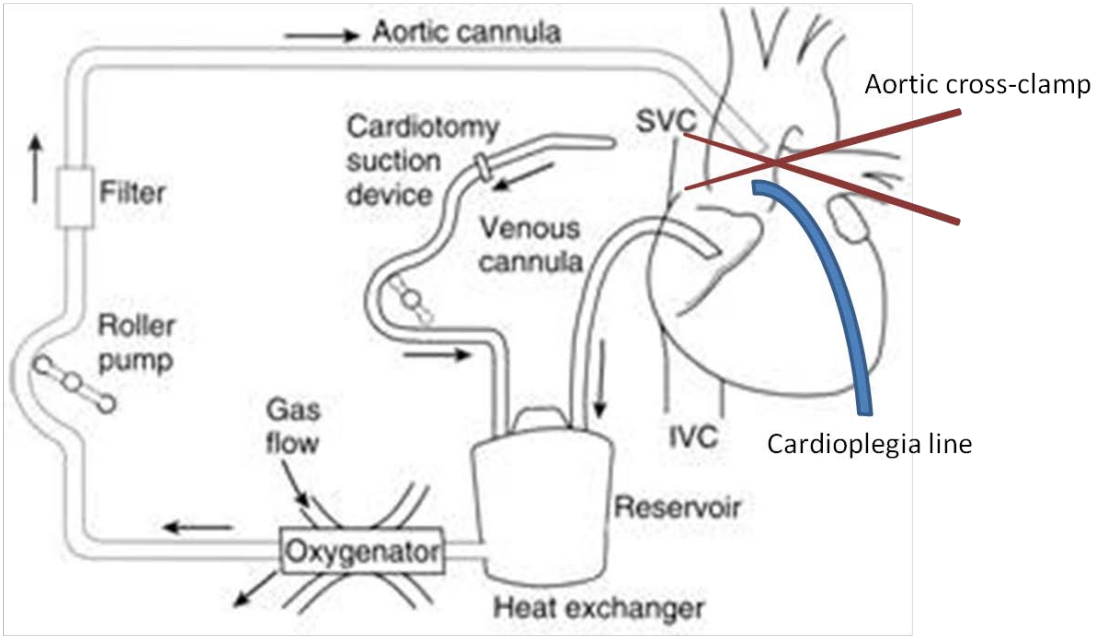


Figure 4 *The cardiopulmonary bypass circuit consisting of a venous reservoir, oxygenator and roller pump connected by right atrial and aortic cannulae. Note also the cardiotomy suction device which is used to return shed blood in the tissue space directly into the venous reservoir.*

In addition to surgical trauma, the inflammatory response to cardiac surgery is often more pronounced because of two unique features of cardiac surgery – CPB and aortic cross-clamping. Blood coming into contact with the foreign surface of the CPB circuit is itself a potent stimulus for inflammation with the activation of humoral factors. It occurs within minutes of the onset of CPB and is characterised by both humoral and cellular activation of the innate immune system (Butler et al. 1993). Both cellular (neutrophils and monocytes) and humoral (complement cascade, clotting/fibronoylsis cascades as well as the kallikrein-bradykinin systems) are activated rapidly. This subsequently triggers the release of cytokines and chemokines such as IL-8 and

MCP-1 which are dependent on the complement activation as a result of surface contact (Lappegard et al. 2004). These factors serve to amplify the signal to the blood cells such as neutrophils and monocytes.

The effect of CPB on the inflammatory response may be better appreciated when the inflammatory response to the procedures which require CPB ("on pump" surgery) are compared with those that do not require cardiopulmonary bypass ("off pump" surgery). The pattern of cytokine release has been found to be different in the two situations. Generally speaking, there is a greater magnitude and earlier release of cytokines in the "on pump" surgical group than in the "off pump" surgical group (Greilich et al. 2008). Proteonomic studies have also shown different gene profiles, with significantly increased cytokine responses especially pro-inflammatory, with higher levels of IL-1β, IL-6, IL-8 and TNF-α in the "on pump" group than in the "off pump" group (Mei et al. 2007).

Another factor contributing to the inflammatory response in CPB is the cardiotomy suction device which is used during "on pump" cardiac surgery cases to return the shed mediastinal blood directly into the venous reservoir of the CPB circuit in order to minimise blood loss. The shed blood in tissue spaces is a rich source of inflammatory cytokines. This allows inflammatory mediators, including products of cellular necrosis, to return directly into the circulation. This phenomenon has been linked to increased inflammatory responses in CPB and, more importantly, the use of cardiotomy suction has not been shown to reduce the need for allogeneic blood transfusions postoperatively (Lau et al. 2007).

As the heart is arrested during cardiac surgery so that the operation may be performed, it undergoes a period of ischaemia which is followed by reperfusion when the aortic cross clamp is removed. Myocardial IR due to aortic cross clamping plays a

role in causing the inflammatory response. The myocardium is a potent source of inflammatory mediators (Gasz et al. 2006). Not only does reperfusion bring oxygen, but it also results in oxidative stress to the already damaged tissue. While significant progress has been made in protecting the heart through the use of hypothermia, metabolic supplements and controlled reperfusion, there is still a degree of acute inflammatory reaction which can be deleterious and this is known as IR injury. Reperfusion initiates the inflammatory process. The role of TGF-β superfamily members in the setting of IR of the heart will be discussed later (see section 4).

Blood flow during CPB is non-pulsatile with a relatively low mean arterial pressure. This non-physiological flow pattern can lead to relative organ hypoperfusion, such as in the splanchnic bed. This may lead to breakdown of the gut mucosal barrier and the transmigration of gut bacterial organisms resulting in the release of bacterial toxins, including LPS, into the systemic circulation. However, in CPB with pulsatile flow, serum endotoxin levels were found to be lower (Watarida et al. 1994). This low level of endotoxaemia is also thought to play a role in contributing to the whole body systemic inflammatory response.

Thus in broad terms, the inflammatory response associated with cardiac surgery may be attributed to surgical trauma, CPB and organ ischaemia, especially involving the heart. In addition, low level of endotoxaemia may also be contributing due to the non-pulsatile flow and relative gut ischaemia, but the clinical importance of this finding is not entirely clear.

3.2 Pattern of the inflammatory response in cardiac surgery

Contact activation of humoral factors in the blood due to CPB is a unique feature of the inflammatory response to cardiac surgery and occurs within minutes of starting CPB. This involves interaction and cross-amplification of coagulation, Kallikrein-

bradykinin, complement and fibrinolytic cascades. Activating the Hageman factor (factor XII) of the coagulation cascade is an initiating event, leading to the activation of the intrinsic coagulation system and conversion of pre-kallikrein to kallikrein, which in turn results in the production of bradykinin and plasmin from plasminogen (part of the fibrinolysis pathway) (Pintar and Collard 2003). The complement factors are also activated by factor XII. Activated complement factors such as C3a and C5a appear in the blood shortly after commencement of CPB. In addition, protamine given at the end of CPB to neutralise heparin forms a complex with heparin and activates the complement system via the classic pathway (Bruins et al. 2000). Activated complement factors serve to amplify the inflammatory response through the activation of neutrophils, platelets and monocytes. This has been implicated in causing acute lung injury and pulmonary edema following cardiac surgery (Gillinov et al. 1993). Furthermore, complement factor depletion due to consumption has been thought to render patients more at risk of infection postoperatively. Interestingly, complement activation in “off pump” surgery is largely absent, suggesting contact activation during CPB is the main trigger for complement activation (Hoel et al. 2007). Specific inhibition of complement factors has been shown to improve organ functions in animal models of CPB but has failed to show a clinical benefit through randomised trials (Smith et al. 2011). Serum levels of these factors peak during CPB and activate neutrophils and monocytes, which in turn produce other pro-inflammatory mediators, such as cytokines, to continue the inflammatory response postoperatively.

The cellular immune response is characterised by neutrophil activation and an increase in circulating neutrophil counts. Organ dysfunction, particularly involving the lungs, has been demonstrated from neutrophil sequestration and activation in the tissues. Neutrophil depletion during CPB has been shown to improve lung function postoperatively (Rubino et al. 2011). Cell-mediated cytotoxic and natural killer T cell responses are diminished post CPB (Nguyen et al. 1992). Similarly, there is a

diminished capacity for T cells to proliferate and secrete cytokines such as IFN-γ and TNF-α (Naldini et al. 1995). CD4+ T cell levels are particularly decreased and blood monocytes have decreased membrane HLA expression, suggesting possible reduced antigen presentation ability (Rinder et al. 1997). B cell number does not appear to be affected and antibody production is increased following CPB (Markewitz et al. 1996). Overall, there appears to be a shift from TH1 (pro-inflammatory) to TH2 (immunomodulatory or anti-inflammatory) responses following cardiac surgery (Franke et al. 2006). This is reflected also in the cytokine profile in which there are decreased levels of IL-2 and IL-12, important for TH1 responses and increased levels of IL-10 and TGF-β which are important for TH2 responses (Markewitz et al. 2001). Further, there is a decrease in cytokine response to LPS from blood immune cells following CPB (Borgermann et al. 2007). It is not exactly clear why cell-mediated immune response is diminished but it is likely due to the traumatic effect of CPB on the cells themselves and the effect of serum inhibitory activities, including the presence of anti-inflammatory and immunomodulatory cytokines.

3.3 Cytokines in cardiac surgery

The pattern of cytokine release in cardiac surgery has been quite extensively studied. There is a complex network of cytokines associated with the inflammatory response to CPB. In the early postoperative period, the pattern can be broadly described by two phases, an early pro-inflammatory phase while on bypass, followed by an anti-inflammatory phase occurring early after the termination of CPB (Landis 2009). Cytokines are important regulators in this balancing act of pro-inflammatory and anti-inflammatory responses. Individual cytokines often exhibit both of these functions depending on the cellular context.

IL-1β and TNF-α are two cytokines elevated early during bypass. IL-1β is secreted by activated monocytes and raises body temperature as well as acting as a mediator of

endothelial permeability and vascular tone (Puhlmann et al. 2005). Levels of IL-β correlate with body temperature following cardiac surgery and predict outcomes in those developing ARDS following cardiac surgery (Meduri et al. 1995).

TNF-α is one of the earliest cytokines released in sepsis but in cardiac surgery, serum TNF-α has not been consistently found to be elevated. The level in “off pump” surgery is lower than in surgery with the use of CPB (Orhan et al. 2007). A study by Wan and colleagues concluded that the release of TNF-α is primarily the result of myocardial IR and not cardiopulmonary bypass *per se* (Wan et al. 1996). But other studies have shown that TNF-α levels post-cardiac surgery correlated with serum endotoxin level and may be a result of gut bacterial transmigration. TNF-α increases adhesion molecules on leukocytes and endothelial cells and works synergistically with neutrophil surface integrin expression to cause neutrophil transmigration (Scholz et al. 2004).

IL-6 is another acute phase cytokine that is elevated following cardiac surgery (Greilich et al. 2008), having both pro-inflammatory and anti-inflammatory roles. IL-6 is well characterised for its role in initiating a hepatic acute phase response but it also stimulates glucocorticosteroid production (Steensberg et al. 2003). In an animal model of surgical trauma, the degree of IL-6 increase correlated with surgical stress (Ishibashi et al. 2006). Preoperative levels of IL-6 and CRP can predict major post-operative complications (Amar et al. 2007). IL-6 levels correlate with post-operative lung function (Halter et al. 2005) and are predictive of postoperative atrial fibrillation (Ucar et al. 2007) and acute renal dysfunction following cardiac surgery (Gueret et al. 2009; Musleh et al. 2009).

IL-8 is a chemotactic factor responsible for recruiting neutrophils to the site of inflammation (Hammond et al. 1995). Its level is elevated around the same timeframe

as IL-6, peaking 60 minutes after the removal of the aortic cross clamp in CPB (Kawamura et al. 1999). Its release is complement-dependent and results from surface contact, as isolated CPB has been shown to be able to stimulate its release from blood cells (Gormley et al. 2003; Lappegard et al. 2004). Like, IL-6, the serum levels of IL-8 correlate with post-operative complications such as acute kidney injury (Liangos et al. 2009).

This initial surge in pro-inflammatory cytokines coincides with the period of CPB and peaks shortly after termination of CPB. This is followed by the release of cytokine antagonists, such that the balance between pro-inflammatory and anti-inflammatory factors is usually maintained in the body's response to injury. These antagonists include soluble receptors for TNF-α, IL-1 and IL-6, IL-10 and TGF-β (McBride et al. 1995; Sablotzki et al. 1997). Soluble TNF receptors levels are elevated in major surgery and coincide with the rise in IL-6 at the end of surgery (Sasajima et al. 2009). Higher pre-operative soluble TNF receptor levels have been correlated with a poorer outcome following cardiac surgery (Gelape et al. 2007). The heart following IR is a source of soluble TNF receptors (Wei et al. 2001). On the other hand, serum soluble IL-6 receptor levels do not change with surgery but there is decreased level in the coronary sinus shortly after bypass, suggesting that these are being taken up within cardiac tissue (Wei et al. 2001). IL-10 is an immunoregulatory cytokine that suppresses the synthesis of pro-inflammatory cytokines both *in vitro* and *in vivo* (Moore et al. 2001). IL-10 is produced by TH2 or regulatory T (Treg) lymphocytes (Frazier and Hall 2008), but may also be secreted from monocytes following activation of the haemoglobin scavenger receptor, CD163, after cardiac surgery (Philippidis et al. 2004). Recently, even neutrophils have been shown capable of IL-10 production in the acute setting, thus revealing a hitherto unappreciated role in immunomodulation (Zhang et al. 2009b). A preoperative high level of IL-10 seems to correlate with a higher degree of immunoparalysis following cardiac surgery and is

predictive of more post-operative complications in paediatric cardiac surgery (Allen et al. 2006).

The ratio of pro-inflammatory and anti-inflammatory cytokines can often be used to predict outcomes after cardiac surgery, such as prolonged ventilation (Rothenburger et al. 2003). In mononuclear cells, TNF-α mRNA levels are decreased whereas IL-10 mNRA levels are increased in routine cases. However, in complicated cases, TNF-α mRNA levels do not decrease as much as in routine cases, resulting in a lower IL-10/TNF-α ratio (Duggan et al. 2006). Steroids can also alter the balance between IL-6 and IL-10, resulting in higher IL-10 levels and this in turn seems to correlate with an improved outcome in high risk patients (Weis et al. 2009), but probably not in low risk patients (Volk et al. 2001).

Higher pro-inflammatory cytokine levels are generally associated with a more complicated post-operative course. In a proportion of patients, the pro-inflammatory component may appear to be exaggerated. This form of inflammatory response is commonly known as SIRS. Increasingly, it is recognised that this exaggerated inflammatory response is also associated with a profound anti-inflammatory response, such that there is a delayed immunoparalysis which can make the patient more susceptible to infections and increased mortality and morbidity (Frazier and Hall 2008). This may explain the perplexing situation where the degree of cytokine elevation has been correlated with mortality and complications following cardiac surgery, but that anti-inflammatory treatment has not always been shown to improve survival in clinical trials (Laffey et al. 2002). This profound immunoparalysis has been shown in septic patients and those with MOD (Pachot et al. 2006). CPB is associated with an initial surge in pro-inflammatory cytokines, but the immune cells exhibit an immunosuppressed phenotype following CPB with impaired cell mediated immunity, as previously discussed. Although this may be an adaptive mechanism for the body

to limit the inflammatory response, if this immunoparetic phenotype is prolonged and fails to recover, patients are likely to suffer from infective complications which are associated with significant morbidity and mortality following cardiac surgery. Studies have shown that while there is a surge in circulating cytokine levels, the cells have a reduced ability to respond to endotoxin challenge (Borgermann et al. 2007). Elevated cytokine levels may be responsible for the downregulation in immune cells, especially monocyte function following cardiac surgery (Strohmeyer et al. 2003). Recently, it was shown that elevated serum TNF-α level led to down-regulation of monocyte TLR4 expression (Tsai et al. 2009). The degree of immunoparalysis may be directly related to the severity of the initial pro-inflammatory response, although the mechanism for this depression has not been fully elucidated.

3.4 TGF-β superfamily members and CPB

In the general cytokine response in inflammation, TGF-β is thought to be an immunosuppressive cytokine but studies that have examined its release in the setting of cardiac surgery has been limited. Serum levels of immunosuppressive cytokines IL-10 and TGF-β was examined by Sablotzki and colleagues. The authors found an increase in the circulating levels of these cytokines at time of weaning off CPB and peaking at the time skin closure (usually one hour from the time of coming off bypass) (Sablotzki et al. 1997). No studies have examined members of either the BMP or activin/inhibin families.

The known initiators of activin A release are present in cardiac surgery and include surgical trauma, myocardial IR injury, endotoxin release due to gut bacterial translocation and cytokines such as IL-1β and TNF-α (Asimakopoulos 2001). Furthermore, endogenous activators of the TLRs are increased following cardiac surgery. Szerafin and colleagues found highly elevated levels of HSP27, HSP60, HSP70 and HSP90α in CPB compared with “off pump” surgery (Szerafin et al. 2008).

Becker and colleagues found a close correlation between HSP70 levels and degree of myocardial damage and white cell count following open heart surgery (Becker et al. 2007). However, TLR expressions in immune cells are known to be down-regulated as a result of CPB (Hadley et al. 2007). Given the involvement of activin A in large number of inflammatory disorders, as outlined above, it is possible that activin A may also be involved in the cytokine response to cardiac surgery. Activin A levels have not been previously measured in CPB due to the need for heparin administration in cardiac surgery. As discussed previously in section 1.4.1, heparin increases circulating levels of activin A and follistatin, concurrently. As the currently utilised ELISA assay does not distinguish between free and follistatin bound activin A, this has hindered efforts to identify the release pattern of activin A in cardiac surgery.

4. Myocardial ischaemia reperfusion injury and inflammation

Ischaemia occurs in cardiac surgery during the period when the heart is arrested. After removal of the aortic cross clamp, the blood flow to the heart is restored. This is the reperfusion phase. It has been increasingly recognised that reperfusion not only brings the necessary nutrients to the organ but also potentiates the inflammatory response that is initiated by the ischemic tissue (Yellon and Hausenloy 2007). Cellular injury does not occur as a result of ischaemia alone. Uncontrolled reperfusion exacerbates ischaemic damage. This can result in arrhythmia, myocardial stunning (transient dysfunction) and, worst of all, myocardial infarction (Moens et al. 2005). Many studies have shown that optimised reperfusion strategies result in reduced myocardial damage compared with uncontrolled reperfusion. This section will review the pathophysiology underlying IR injury and the potential involvement of TGF-β superfamily members in myocardial IR.

4.1 Pathophysiology of IR injury

The cellular changes which occur at the time of reperfusion are important determinants of cardiomyocyte survival (Carden and Granger 2000). Influx of oxygen during reperfusion increases ROS production due to damage to the mitochondrial electron transport chain during ischaemia. This is accompanied by an abrupt increase in cytoplasmic and mitochondrial calcium concentrations. Elevated levels of intracellular calcium have been associated with hyper-contracture of cardiac muscle as well as mitochondrial dysfunction in which there is diminished mitochondrial membrane potential and opening of MPTP (Piper et al. 2004). Calcium overload has been shown by many studies to precede irreversible myocardial damage (Imahashi et al. 2005). In addition, rapid restoration of physiological pH has also been linked to the opening of MPTP (Lemasters et al. 1996). Opening of MPTP is thought to be an important initiating event of cell death (Borutaite et al. 2003). It dissipates the

mitochondrial membrane potential, resulting in depleted ATP stores. Cell death during IR can be as a result of necrosis, apoptosis and autophagy (Murphy and Steenbergen 2008). Although the relative contribution of each to the overall infarct size is still debated, it is clear that an inflammatory response is an important component to IR injury. Necrotic cells release DAMPs, including HMGB1 and HSPs, and this can result in recruitment of inflammatory cells and endothelial injury. Neutrophils are recruited to the site during the first six hours and interact with endothelial cells that result in their transmigration across the vasculature into the tissue **(Figure 5).**

The microvasculature is an important site for the inflammatory response. Endothelial cells line the inner surface of blood vessels and are a dynamic structure that is essential for vascular homeostasis (Carden and Granger 2000). In response to ischaemia and reperfusion, there is a rapid up regulation of proteins such as endothelin, thromboxane A2 and down-regulation of NO and prostacyclin (Carden and Granger 2000). These changes turn the endothelial surface into a pro-inflammatory surface, which loses its normal anticoagulant and vasodilatory properties. Activated leukocytes adhere to the surface and cells swell and lift off from underlying basement membrane. In the arterioles, there is a loss of endothelium-dependent NO-mediated relaxation which is partly the result of ROS production (Harrison 1997). Mice lacking adhesion molecules are also less prone to these changes (Banda et al. 1997). There is also increased permeability of the capillary bed which seems to be mediated by diminished NO production (Harris 1997). In addition, there is reduced capillary blood flow either due to plugging by adherent leukocytes or the swollen endothelial cells themselves, potentially resulting in further ischaemia to the heart (no re-flow phenomenon). The response is also amplified by the resident macrophages and mast cells (Kubes and Granger 1996).

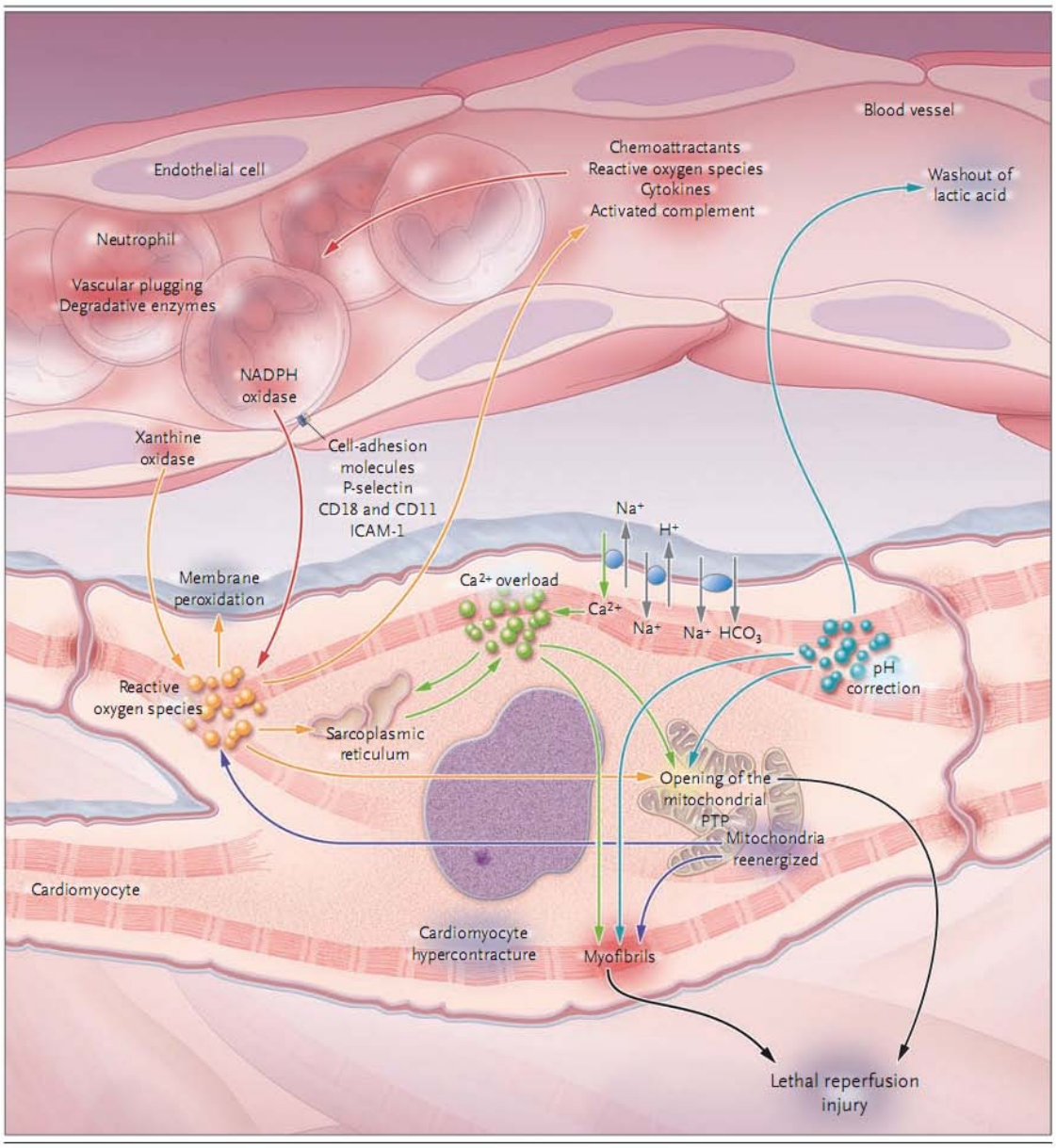


Figure 5 Myocardial IR injury. Cellular mechanisms for injury include ROS production by cardiomyocytes, endothelial cells and neutrophils; calcium overload and opening of MPTPs. From (Yellon and Hausenloy 2007)

The endothelium is rich in xanthine oxidase. Ischaemia promotes the conversion of the NAD-reducing dehydrogenase form of this enzyme to the oxygen-reducing oxidase form. Hypoxanthine accumulates during ischaemia and when oxygen is re-introduced during reperfusion, xanthine oxidase catalyses the reaction between hypoxanthine and oxygen to form hydrogen peroxide. An initial burst of ROS

production by the ischaemic cells following reperfusion has been consistently demonstrated (Murphy and Steenbergen 2008). This is sustained by the respiratory burst by adherent neutrophils, which produces more ROS. Reducing leukocyte-endothelium interactions by blocking adhesion molecules reduces ROS production. Complement factors are also activated as a result of IR, with deposits of complement fragments on the microvasculature. They are chemotactic for neutrophils and cause neutrophil activation and adhesion molecules expression (Lucchesi 1994). There is also a significant body of literature to suggest that diminished NO production correlates with the degree of endothelial dysfunction. NO is critical in maintaining vascular homeostasis under normal conditions, as it can effectively scavenge low levels of superoxide in cells, cause arteriolar relaxation by activating guanylate cyclase in smooth muscles, prevent platelet aggregation and minimise leukocyte adhesion (Carden and Granger 2000). The balance is tipped during IR in which there is a massive production of superoxide and hydrogen peroxide. NO depletion during ischaemia reperfusion converts the resting endothelium into a pro-inflammatory phenotype.

Neutrophil depletion (Litt et al. 1989), the use of antibodies against adhesion molecules (Zhao et al. 1997) and complement inhibition (Vakeva et al. 1998) have all been shown to reduce IR injury. More recently, TLR signalling has been shown to be involved in myocardial IR. Using genetic knock out or pharmacological inhibition of TLR4 signalling reduces infarct size following reperfusion (Chong et al. 2004; Shimamoto et al. 2006). This suggests that sensing of DAMPs by TLR is an important trigger for the inflammatory response to IR.

Severe ischaemia reperfusion can result in remote organ dysfunction and possibly result in MOD. Often respiratory insufficiency is the first sign of such an event. This is characterised by increased pulmonary capillary permeability and neutrophil-rich

alveolar fluid. This is followed by other organ failures such as the liver and the kidneys and coagulation and immune dysfunction, resulting in disseminated intravascular coagulation and immunoparalysis (Carden and Granger 2000). The contributing factors to these systemic involvements are activated leukocytes and inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Aird 2003). There is a balance of both pro-inflammatory cytokines and ant-inflammatory cytokines and soluble receptors, resulting in a complex regulatory network as discussed in the previous section. Cytokines such as TNF-α may be the key mediators of the systemic effects of IR including lung neutrophil sequestration and capillary leakage (Welborn et al. 1996).

4.2 Role of TGF-β superfamily members in IR induced inflammation

Cardiac surgery causes elevated serum levels of anti-inflammatory TGF-β and IL-10 which occur after the initial surge in the serum levels of pro-inflammatory cytokines (Sablotzki et al. 1997). In cultured rat cardiomyocytes and bovine heart endothelial cells, TGF-β is able to decrease the production of free radicals by reducing the expression of xanthine oxidase when given in conjunction with inflammatory stimuli (Flanders et al. 1997). This suggests that TGF-β may be part of the counter-regulatory mechanism in limiting IR injury of the heart. However, at the protein level, only the latent form of the protein is increased but not the active form (Mehta et al. 1999). Platelets are a rich source of TGF- β and have been found to be protective in myocardial IR (Mehta et al. 1999). TGF-β appears to be able to reduce apoptosis in cultured cardiomyocytes and up-regulation of anti-apoptotic protein Bcl-2 (Grunenfelder et al. 2002; Yang et al. 1999). Further studies showed that the anti-ischaemic effect may be abolished by blocking signalling through p38 and ERK1/2 pathways (Baxter et al. 2001; Mocanu et al. 1998). TGF-β can also up-regulate inducible NOS and protein kinase B pathway and suppress MMP-1 up-regulation (Chen et al. 2001, 2003). However, it appears that sustained up-regulation of TGF-β

is associated with later ventricular dilatation and fibrosis consistent with the known fibrotic effects of TGF-β (Ikeuchi et al. 2004).

A number of other TGF-β superfamily members, notably in the BMP subfamily, have also been shown to be protective in IR injury. BMP-7 (also known as osteogenic protein-1) has been shown to reduce cerebral infarcts in transient carotid occlusion of rats and renal IR injury (Perides et al. 1995; Vukicevic et al. 1998). Further, BMP-6 has been found to be released by neurons into the interstitial space after mild reversible ischaemia and reduces brain IR injury (Martinez et al. 2001; Wang et al. 2001). Both BMP-6 and BMP-7 have been studied in the brain but not in relation to myocardial IR. However, BMP-2 has been shown to decrease apoptosis in serum deprived cardiomyocytes (Izumi et al. 2001) and transfection of SMAD1, a downstream BMP signalling molecule, has been shown to decrease myocardial IR injury (Masaki et al. 2005). This is thought to be partly mediated by its effect on the anti-apoptotic protein, Bcl-xL (Masaki et al. 2005). BMP-4, on the other hand, has been shown to be detrimental in cardiac IR by promoting apoptosis (Pachori et al. 2010). *In vivo* treatment with noggin or dorsomorphine, both of which are inhibitors of BMP signalling, was associated with reduced myocardial infarct size in this study.

The activin subfamily of proteins has also been implicated in IR injury. However, the effects of activin A seem to be more complex than those of the TGF-βs and BMPs. Studies have demonstrated both protective and harmful effects of activin A in IR injury. It has been shown that in transient cerebral ischaemia, activin A subunit mRNA levels are up regulated (Mukerji et al. 2007) and exogenous administration of activin A is associated with reduced cerebral infarct volume (Mukerji et al. 2009). In renal IR injury, activin A was also found to be up-regulated and administrating follistatin, as a high affinity binding protein for activin, was associated with significantly improved histological appearance of kidney tubules and creatinine

clearance (Maeshima et al. 2001). In the heart, it was shown that IR is associated with elevated intra-myocardial levels of activin A and ischaemic pre-conditioning, a manoeuvre associated with reduced IR injury, is associated with reduced activin A expression in the heart (Zhang et al. 2009a). However, a recent study which examined the role of acitvin A in myocardial IR concluded that activin A is in fact protective in this setting (Oshima et al. 2009). These authors demonstrated that activin A over-expression reduced infarct size *in vivo* and that *in vitro*, similar to TGF-β, activin A had an anti-apoptotic effect through its ability to up-regulate cytoplasmic Bcl-2 levels (Oshima et al. 2009). Although many of TGF-β superfamily members share the same intracellular signalling pathways, these conflicting results suggest that the function of these proteins may be dependent on the cellular context. Further studies are therefore required to elucidate the role of these proteins in IR.

5. Concluding remarks, statement of aims and hypotheses

In conclusion, activin A has been identified as a novel cytokine which may play an immunoregulatory role in acute inflammation. It is elevated in a broad range of inflammatory conditions and has also been implicated in tissue fibrosis. Cardiac surgery is associated with non-specific whole body inflammatory responses due to the use of CPB and myocardial IR injury. CPB is associated with significant contact activation of humoral factors leading to recruitment of effector cells such as neutrophils. However, some aspects of the cell-mediated immunity are diminished as a result of CPB and the underlying mechanisms are still not entirely clear. An inflammatory response has been consistently demonstrated in myocardial IR. Controlled reperfusion of ischaemic myocardium has been shown to improve myocardial viability. Decreasing the inflammatory process, for example by inhibition of TLR signalling or reducing neutrophil endothelial cell interaction, has been shown to reduce myocardial infarct size. Furthermore, a number of TGF-β superfamily members, including activin A, have been implicated in IR. The role of activin A in cardiac surgery has not been previously explored, nor have there been any studies that have examined the therapeutic potential of follistatin in neutralising any detrimental effect of activin A in the setting of cardiac surgery. The overall aim of this thesis is to study the release pattern and function of activin A and follistatin in CPB and myocardial IR. An additional aim is to identify the potential sources of activin A during acute inflammation.

In *Chapter 2*, the release pattern of activin A and follistatin in a sheep model of CPB is presented.

In *Chapter 3*, the expression pattern of activin A and follistatin is studied in a mouse model of *in situ* myocardial IR injury as well as *in vitro* cardiomyocyte culture experiments and the potential of follistatin in reducing IR injury is assessed.

In *Chapter 4*, circulating neutrophils are identified as a potential source of preformed activin A and the mechanisms of its release from neutrophils are studied.

In *Chapter 5*, the overall significance of the findings of this thesis is discussed and possible directions for future research are presented.

CHAPTER 2

PATTERN OF ACTIVIN A AND FOLLISTATIN RELEASE IN A SHEEP MODEL OF CARDIOPULMONARY BYPASS

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Design and conduct of the experiments, data analysis and final write up	65%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Phillips DJ	Design of experiment, data analysis, manuscript revision	
McMillan J	Experimental work	
Bedford P	Experimental work	
Goldstein J	Experimental work	
Wu H	Experimental work	10%
Hedger MP	Design of experiment, data analysis, manuscript revision	
Smith, JA	Design of experiment, data analysis, manuscript revision	

Candidate's signature [redacted] **3/3/2012**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Monash Institute of Medical Research**

Phillips, DJ

McMillan, J

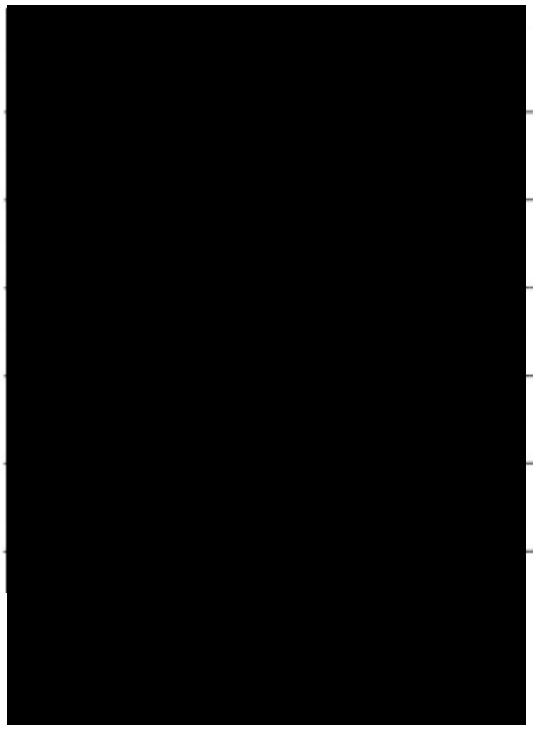
Bedford, P

Goldstein, J

Wu, H

Hedger, MP

Smith, JA



Cytokine 54 (2011) 154–160

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Pattern of activin A and follistatin release in a sheep model of cardiopulmonary bypass

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ARTICLE INFO

Article history:
Received 9 July 2010
Received in revised form 11 December 2010
Accepted 2 January 2011
Available online 12 February 2011

Keywords:
Activin A
Follistatin
Cardiopulmonary bypass
Inflammation

ABSTRACT

Objective: Activin A, a member of transforming growth factor-β superfamily, has been established as a critical cytokine released early in endotoxemia and other inflammatory syndromes. The release of activin A and its binding protein, follistatin during cardiopulmonary bypass (CPB) has not been previously reported. Our study aimed to define the pattern of activin A and follistatin release in a sheep CPB model.
Methods: Control group consisted of left thoractomy alone ($n = 6$). CPB was performed using either unfractionated heparin ($n = 6$) or lepirudin ($n = 6$) as anticoagulant. Unlike heparin, lepirudin does not cause activin A and follistatin release on its own. Serum samples were assayed for activin A, follistatin, tumour necrosis factor-α and interleukin-6.
Results: Compared with the control group, CPB using lepirudin was associated with a biphasic release of activin A. The first peak occurred within the first hour of CPB and a second peak occurred within the early post-operative period, coincident with a large release of follistatin. Close correlation was found between follistatin and IL-6 in the control and lepirudin groups, indicative of a role for follistatin in the acute phase response. In contrast to the control and lepirudin groups, CPB using heparin resulted in a concurrent release of activin A and follistatin.
Conclusions: CPB is a trigger for the release of biologically-active free activin A into the circulation, at levels considerably greater than that induced by surgery alone. Triggering release of this critical inflammatory cytokine suggests that activin A may contribute to the adverse outcomes associated with systemic inflammation in cardiac surgery.

1. Introduction

Cardiopulmonary bypass (CPB) is associated with a non-specific generalised inflammatory response [1]. It occurs within minutes of the onset of CPB and is characterised by both humoral and cellular activation of the innate immune system. In a small proportion of patients undergoing CPB, this response may be overwhelming and result in multi-organ dysfunction (MODs) [2]. This form of inflammatory response, commonly known as the systemic inflammatory response syndrome (SIRS), has many features in common with sepsis and septicaemia. Cytokines such as tumour necrosis factor-α (TNF-α) and interleukin (IL)-8 are rapidly released [3,4]. It is not completely understood why in certain patients this inflammatory response is exaggerated. Cytokines that are released early are likely to be the key orchestrators of this whole body inflammatory response.

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily of proteins, has been recently shown to be a critical cytokine released early in the inflammatory response to endotoxemia [5,6]. In a sheep model of endotoxemia, activin A was found to be released very early, coinciding with the release of TNF-α and preceding IL-1β and IL-6 [7]. Studies in mice indicated that this release of activin A was the result of activation of the Toll like receptor-4 (TLR-4) signalling pathway [5]. Furthermore, mortality in mice after challenge with a lethal dose of lipopolysaccharide (LPS) was significantly reduced if activin A was blocked with its binding protein follistatin [5]. Follistatin recognises a number of TGF-β superfamily members but has highest affinity for activin A [8]. Two forms of follistatin exist through alternative splicing. Follistatin 288 is constitutively able to attach to heparan-containing proteoglycans on the cell surface, whereas follistatin 315 is the circulating form and attaches to proteoglycans only after it has bound to actvin A [9]. Follistatin is also released during endotoxemia but its serum level peaks at a later time point, probably as part of a short loop feedback mechanism [7].

A number of studies have shown elevated levels of potential endogenous ligands of the TLR-4 signalling pathway, such as heat

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doi:10.1016/j.cyto.2011.01.001

shock proteins and high mobility group box protein, during cardiac and thoracic surgery [10–12]. Clinical studies have found elevated serum levels of activin A level in patients with angina pectoris and congestive heart failure [13,14]. Recently activin A has also been shown to be involved in myocardial ischaemia reperfusion injury [15]. Both ischaemia reperfusion and the use of CPB are important triggers for the inflammatory response in cardiac surgery. No study has examined whether CPB *per se* is a trigger for activin A or follistatin release into the circulation.

The use of unfractionated heparin during CPB releases a number of heparin-binding growth factors, of which activin A and follistatin are prime examples. Studies in sheep and human have established the existence of a pool of extracellular, cell surface heparan proteoglycan bound activin A-follistatin complexes that are releasable upon systemic administration of heparin [16,17]. By virtue of structural similarity, heparin is able to displace these complexes into the circulation. In contrast, hirudin-based anticoagulants, such as lepirudin, do not release activin A or follistatin (Phillips et al., unpublished data). In our sheep model we therefore used lepirudin as anticoagulant to assess the release of activin A and follistatin by CPB without the confounding effect of heparin.

Through this study, we aimed to establish, for the first time, the pattern of activin A and follistatin release in isolated CPB using a sheep model. A supplementary aim was to identify the potential source of activin A by comparing the release pattern of activin A in CPB *in vivo* with a simulated extra-corporeal circulation running isolated blood *in vitro*.

2. Materials and methods

2.1. Animals and general experimental details

All experiments were conducted in accordance with the National Health and Medical Research Council of Australia (NHMRC) Code of Practice for the Care of Animals for Scientific Purposes (2004) and were approved by the Monash Medical Centre Animal Ethics Committee.

Adult ewes (median weight 58 kg, range 47–82 kg) were divided into three groups. One group served as surgical controls in which a left thoracotomy was performed without CPB ($n = 6$). CPB was carried out in the other two groups using unfractionated heparin ($n = 6$) and lepirudin ($n = 6$) as anticoagulant. There was one death in each group earlier on in the series due to difficulties with ventilation.

2.2. Lateral thoracotomy and CPB

A left lateral thoracotomy was used to provide access to the heart for cannulation for CPB [18]. Briefly, using 6 mg/kg propofol (Pharmatel Fresenius Kabi) induction and 2% isoflurane (Abbott Australasia) maintenance, the ewe was anesthetised and placed in a left lateral position. Arterial and venous cannulae were inserted into the carotid artery and jugular vein using a cutdown technique for pressure monitoring. The chest was opened in the fourth intercostal space. A 40/36 French two-staged venous cannula (Edwards Lifesciences) was inserted into the right atrium and secured with purse string suture. A 22 French aortic cannula (Edwards Lifesciences) was inserted into the proximal descending aorta. CPB was established using 1 L of crystalloid priming solution. Flow was maintained at above 3 L/min with a perfusion pressure between 50 and 70 mm Hg. The total bypass time was two hours under normothermic conditions. The heart lung machine consisted of a venous reservoir, roller pump (Cobe Cardiovascular) and membrane oxygenator (Medtronic Australia) connected by non-coated tubing. Following bypass, the chest was closed and the ewe was allowed to recover for 24 h. Blood samples were taken at baseline (time point 1), pre-bypass (time point 2), every 30 min during bypass (time points 3–6), every 2 h in the first 8 h of post-operative period (time points 7–11) and every 4 h (time points 12–15) in the remaining study period. Serum derived from these samples was stored at −20 °C until assayed for activin A, follistatin, TNF-α and IL-6 concentrations.

2.3. Heparin and lepirudin anticoagulation

Unfractionated porcine heparin (Pfizer Australia) was given as an intravenous bolus at 300 U/kg prior to CPB. Activated clotting time (ACT) was monitored every 15 min to maintain a level above 480 s during CPB. Extra doses of heparin (5000 U) were given if ACT fell below 480 s. Protamine (Sanofi Aventis) (1 mg per 100 U heparin) was given at the end of CPB to neutralise heparin.

Lepirudin (Pharmion) was given as per published protocol [19]. A manual ecarin time was used to monitor serum level of lepirudin as previously described [20]. Serum lepirudin level was maintained at 3.5–4.5 μg/ml while on CPB.

2.4. Simulated extracorpereal circulation

In simulated extracorporeal circulation, 500 ml of blood was collected from healthy adult ewes ($n = 3$). Five hundred ml of crystalloid prime was added to give a final volume of 1 L. This was allowed to circulate in the heart lung machine for 2 h. Blood samples were taken at baseline and every 30 min during the run. EDTA was used as anticoagulant. Serum was derived from individual samples and stored at −20 °C until assayed as described previously.

2.5. Correction for haemodilution

Haemodilution during CPB occurred as a result of the priming volume in the CPB circuit. The serum levels of cytokines in CPB were not directly comparable to that taken in the control case [21]. In order to correct for this haemodilution, cytokine levels were determined in relation to the packed cell volume (PCV) of the blood taken at the time:

PCV corrected cytokine level
= measured cytokine level
× baseline PCV/PCV at time of sampling

2.6. Immunoassays

Activin A was measured by ELISA as previously described [22]. The assay measures both free and follistatin bound activin A and has been validated for ovine biological fluids. The mean sensitivity in the assays was 0.013 ng/ml. The mean intra- and inter-assay coefficient of variation (CV) were 4% and 6%, respectively.

Follistatin was measured by RIA as previously described [23]. The assay measures both free and bound forms of follistatin. The assay employs purified heterologous bovine follistatin as standard and uses iodinated bovine follistatin as tracer. The assay sensitivity was 2.7 ng/ml, the mean ED50 was 13.3 ng/ml and the intra- and inter-assay CV values were 6.4% and 10.2%, respectively.

TNF-α was measured by an ELISA specific for the ovine form as previously described [24]. The standard employed was ovine recombinant TNF-α (Centre for Animal Biotechnology [CAB], University of Melbourne, Australia). The sensitivity of the assay was 0.5 ng/ml, and the intra- and inter-assay CV values were both 10%.

IL-6 was detected by ELISA as previously described, using antibodies specific to ovine IL-6 (Epitope Technologies, Melbourne,

Australia).[24] The standard was ovine recombinant IL-6 from CAB. The sensitivity of the assay was 0·2 ng/ml and the intra- and inter-assay CV values were 10% and 12%, respectively.

2.7. Statistical analyses

Data are expressed as mean ± standard error of the mean. Cytokine responses were estimated using an area under the curve (AUC) function as a measure of total cytokine released within the study period. Peak values were expressed as fold changes from baseline values. Student's *t*-test and the Mann–Whitney test were used where appropriate for comparing means between two groups of continuous data. A p value $\leqslant 0.05$ was considered statistically significant. Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for the graphing and statistical analyses.

3. Results

3.1. Surgery alone causes a small release of activin A and follistatin

A previous study has shown that plasma follistatin concentrations increased from about seven hours after sham castration in sheep as a result of surgical trauma alone [25]. Serum activin A concentrations were not measured in that study. In the current study, surgical trauma alone caused a fourfold increase in serum activin A concentrations compared with baseline (Fig. 1A and D). Concentrations peaked within the first 4 h of surgery and were followed by a delayed but more sustained release of follistatin (Fig. 2A and D). This suggests that both activin A and follistatin may play a role in the acute phase response following surgical trauma.

3.2. CPB using lepirudin as anticoagulant results in biphasic release of biologically active free activin A

We assessed the heparin-independent effect of CPB on activin A and follistatin serum levels using lepirudin as the anticoagulant. Activin A was released rapidly following CPB, peaking within the first hour of bypass (Fig. 1B and D). This was followed by a smaller secondary peak in the early post-operative period (between 6 and 8 h after completion of operation). This pattern was similar to that seen in endotoxemia models of activin A release in sheep and mice [5,7]. A robust follistatin response was seen in the early post-operative period, with a higher peak than that in the control, suggesting a combined effect of CPB and surgical trauma (Fig. 2B and D, Table 1).

3.3. Heparin initiates a concurrent release of activin A and follistatin

Compared with both the control and the lepirudin groups, CPB using heparin as the anticoagulant resulted in a very rapid and concurrent release of activin A and follistatin, an effect due to the presence of heparin (Fig. 1C and C). Activin A concentrations were elevated for the whole duration of the bypass and slowly declined in the early post-operative period following the administration of protamine for heparin reversal. Given the concurrent pattern of activin A and follistatin release, this most probably represents the release of activin and follistatin complexes rather than free activin A into the circulation.

3.4. TNF-α and IL-6 levels in cardiopulmonary bypass

In contrast to many clinical studies [26], we did not find a significant change in circulating TNF-α concentrations with either surgery or CPB (Fig 3A). However, IL-6 showed an increase in

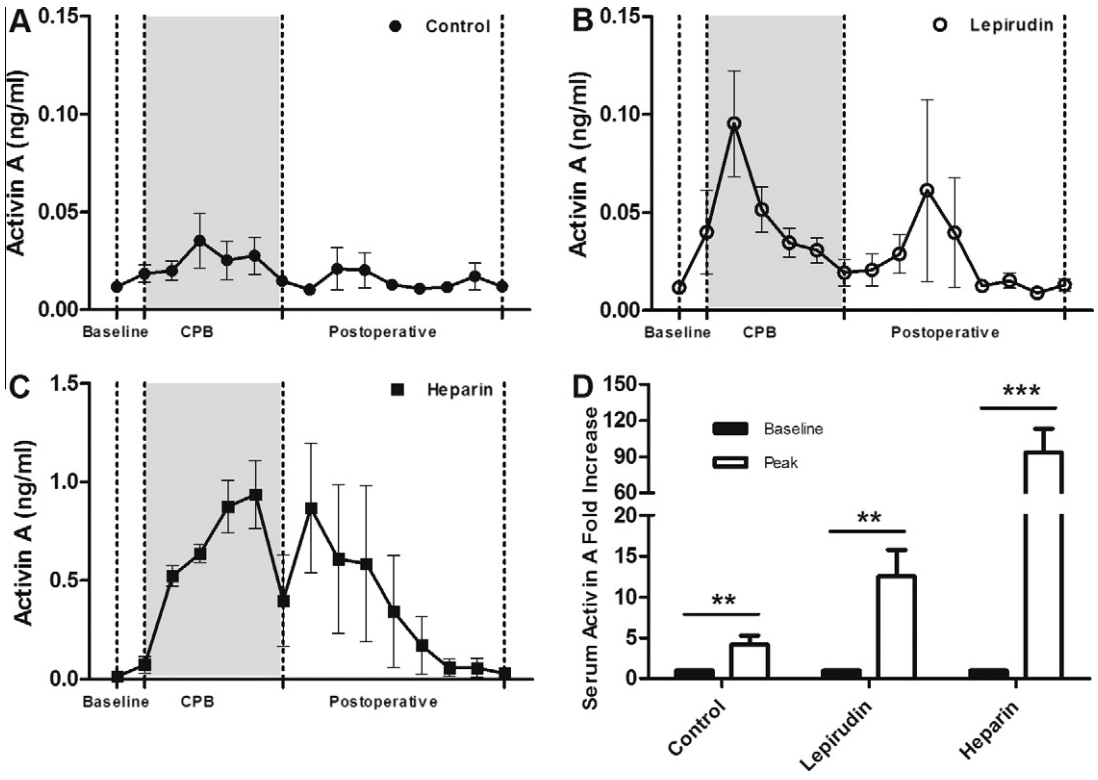

Fig. 1. Serum activin A levels (mean ± SEM) in the control group (●) A, lepirudin-CPB group (○) B, and heparin-CPB groups (■) C over 15 time points (1, baseline; 2, prebypass; 3–6, CPB; 7–15, post-operative period 24 h). Peak activin A levels in each group were compared with baseline levels and represented as fold changes in D. $^{**}p < 0.01$; $^{***}p < 0.001$; comparisons between baseline and peak values.

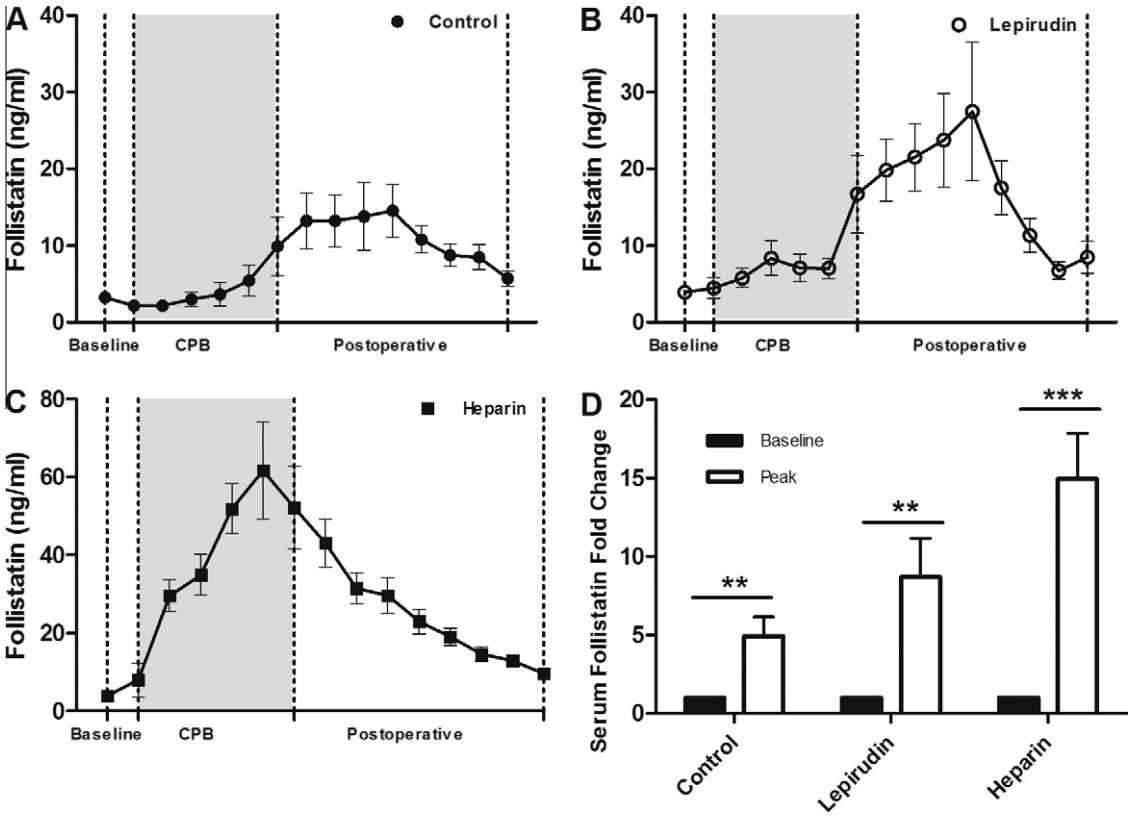

Fig. 2. Serum follistatin levels (mean ± SEM) in the control group (●) A, lepirudin-CPB group (○) B, and heparin-CPB group (■) C over 15 time points (1, baseline; 2, prebypass; 3–6, CPB; 7–15, post-operative period 24 h). Peak follistatin levels in each group were compared with baseline levels and represented as fold changes in D. $^{**}p < 0.01$; $^{***}p < 0.001$; comparisons between baseline and peak values.

Table 1
Total (AUC) and peak activin A and Follistatin levels in the three groups.

	Activin A						Follistatin					
	AUC (ng/ml/h)	±SEM	*p*-value*	Peak (ng/ml)	±SEM	*p*-value*	AUC (ng/ml/h)	±SEM	*p*-value*	Peak (ng/ml)	±SEM	*p*-value*
Control	0.254	±0.046		0.049	±0.013		109.797	±23.240		16.050	±3.830	
Lepirudin	0.460	±0.068	0.037	0.130	0.0371	0.037	183.300	±35.050	0.100	34.037	±9.783	0.100
Heparin	6.486	±2.000	0.007	1.210	±0.258	0.001	384.340	±36.060	0.0001	57.466	±11.140	0.0001

AUC, area under the curve; SEM, standard error of the mean.
* Lepirudin and heparin groups vs. control.

serum concentrations in the post-operative period in both the control and the CPB groups, consistent with its role as an initiator of the acute phase response (Fig. 3B). There was a significant degree of correlation between serum IL-6 and follistatin levels in the control and lepirudin groups ($p < 0.0001$), but not between IL-6 and follistatin in the heparin group (Fig 3C–E). No correlation was found between IL-6 and activin A levels in any of the three groups (data not shown).

3.5. Ex vivo simulated-CPB does not cause significant release of activin A or follistatin

As blood monocytes are known to release activin A following LPS stimulus, we examined whether circulating blood cells may be a source of activin A and follistatin released on CPB *in vivo*. We circulated sheep blood *ex vivo* in a heart lung machine, but activin A or follistatin concentrations in this blood were unchanged (Fig. 4A–B).

4. Discussion

This is the first study to examine the role of activin A and follistatin in CPB. When heparin was used as anticoagulant in CPB, there was a high level of concurrent release of activin A and follistatin. However, it is important to emphasise that heparin releases activin A and follistatin as a complex by displacing them from cell surface heparan-containing proteoglycans. Follistatin binds to activin A with picomolar affinity and this is considered essentially irreversible. Even though there is a large increase in both serum activin A and follistatin levels following heparin administration, as a complex neither of these can be considered biologically active. A limitation of the current assays is that only total serum activin A and follistatin levels can be measured. By using a hirudin-based anticoagulant which does not cause release of activin A or follistatin, we have shown that CPB itself was associated with a biphasic pattern of activin A release and a delayed follistatin response. This pattern has been observed in sheep and mouse models of endotoxemia.[5,7] This suggests that there is a common mechanism

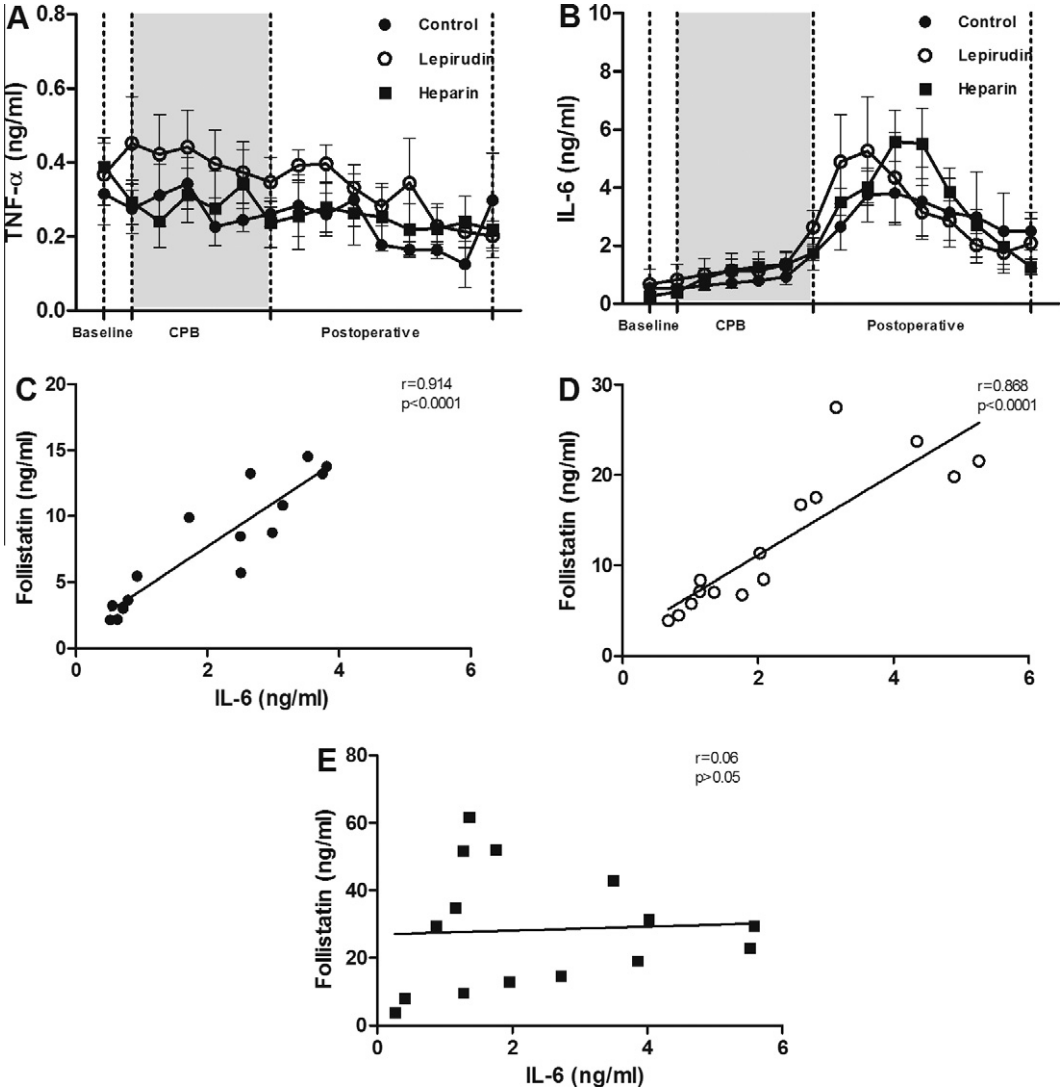

Fig. 3. Serum TNF-α (A) and IL-6 (B) levels in the control group (●), lepirudin-CPB group (○) and heparin-CPB group (■) over 15 time points (1, baseline; 2, prebypass; 3–6, CPB; 7–15, post-operative period 24 h). There were no statistically significant changes amongst the three groups. Positive correlations were found between serum IL-6 and follistatin levels in the control group (C) and the lepirudin-CPB group (D) but not in the heparin-CPB group (E).

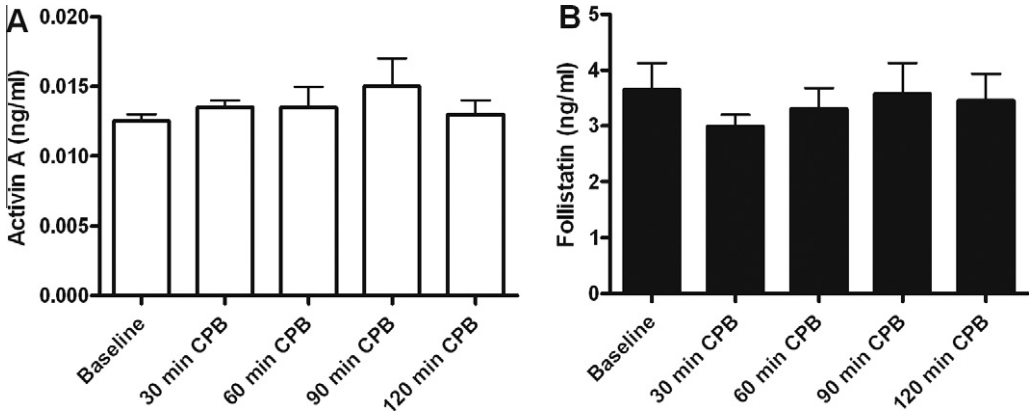

Fig. 4. Serum activin A (A) and follistatin (B) concentrations in the extra-corporeal circuit using sheep blood (450 ml) run for two hours *ex vivo* in a heart lung machine. No statistically significant changes in serum activin or follistatin concentration were noted.

involved in the release of activin A and follistatin in the setting of acute inflammation and an exaggerated release of activin A may be involved in SIRS. In a mouse model of severe endotoxemia, blocking activin A using follistatin was shown to reduce inflammation and improve survival [5]. It remains to be seen whether a similar approach may be used to attenuate CPB-induced inflammation.

The effects of activin A *in vivo* on inflammation and immunity are complex. Accumulating evidence suggests that it has both pro-inflammatory and immunoregulatory effects [6]. During acute inflammation the principal role of activin A appears to be the promotion or amplification of the inflammatory response. It stimulates the nuclear translocation of the inflammatory transcription factor, NFκB and upregulates expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α and inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 in macrophages. Conversely, *in vitro* studies have shown that activin A can antagonise some of the actions of IL-1β and IL-6 including the production of acute phase proteins. It can also regulate the immune response by stimulating regulatory T cell development and maturation of tolerising dendritic cells, as well as inducing a type 2-immune response in lymphocytes and macrophages [27].

The cytokine release profile in cardiac surgery typically has two phases [3]. A pro-inflammatory early phase occurs at the time of CPB with the release of cytokines such as TNF-α and IL-8. This is followed by a late anti-inflammatory phase occurring during the early post-operative period and is characterised by increased serum IL-10 and TGF-β levels [28]. In the present study, the release of activin A was biphasic, with the first peak occurring during the time of CPB, followed by a smaller secondary peak in the early post-operative period, coinciding with the pro-inflammatory and anti-inflammatory phases seen after CPB. This is consistent with the emerging concept that activin A plays a pro-inflammatory role in acute inflammation, but may switch to a more complex immunomodulatory role in later phases of inflammation [6].

In this study, we have found a close correlation between serum IL-6 and follistatin levels. The concept that follistatin is an acute phase protein has been previously described [25]. Moreover, activin binding to follistatin is thought to be the pathway whereby activin A is removed from the circulation to be internalised and degraded [9]. This suggests that the release of follistatin during the acute phase response limits the action of activin A. The peak release of follistatin in CPB coincided with the second peak in the activin A response. The release of follistatin in CPB may also be due to the earlier release of activin A as LPS-injected mice, pretreated with follistatin to block the action of activin A from onset of inflammation, do not display a late peak of follistatin [5]. This highlights the possibility that blocking the first peak of activin A with exogenous follistatin during CPB may potentially reduce the early pro-inflammatory effects of activin A in this setting.

It is still unclear what the cellular sources of activin A and follistatin are during events such as CPB. A number of cell types, including vascular endothelial cells, various epithelia, monocytes/macrophages and dendritic cells have been found to be able to synthesise activin A following inflammatory stimuli [6]. We examined blood cells as a potential source of activin A and follistatin released during CPB. We were unable to detect a significant change in serum activin A or follistatin concentrations when the blood was circulated in a heart lung machine *ex vivo*. This suggests that blood coming into contact with a foreign surface was not adequate on its own to trigger the release of activin A and follistatin from blood cells. Either blood cells are not the major contributors to serum activin A and follistatin levels in CPB or other tissue-derived factors are required to trigger their release.

We did not find a significant difference in TNF-α levels in the three groups. This suggests that CPB *per se* is not a strong stimulus for the release of this cytokine. This is consistent with a previous study which showed that the heart is the major source of circulating TNF-α following cardioplegia arrest [29]. Since the heart was perfused throughout the CPB period, the expectation might be that serum levels of TNF-α would not change significantly compared with the control group.

In conclusion, the release of free activin A occurred in a biphasic fashion, coinciding with the pro-inflammatory and anti-inflammatory phases of the whole body response to CPB. This is independent of the effect of unfractionated heparin which caused the release of activin A-follistatin complexes. Follistatin was released around the time of the second phase of the activin A response and was correlated closely with serum IL-6 levels. These data has established that CPB is a trigger for the release of biologically active free activin A into the circulation and the release of this critical inflammatory cytokine early in the setting of CPB suggests that it may be an important orchestrator of the whole body inflammatory response to cardiac surgery.

Acknowledgements

The authors would like to thank Sue Hayward for her technical expertise with the ELISA assays and Nikolai Faizoulline for assistance with animal procedures. The study is supported by grants from Australasian Society of Cardiac and Thoracic Surgeons, the Royal Australasian College of Surgeons, Southern Health and the National Health and Medical Research Council (NHMRC) of Australia. It was also supported in part by the Victorian Government's Operational Infrastructure Support Program.

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CHAPTER 3

REGULATION AND ACTIONS OF ACTIVIN A AND FOLLISTATIN IN MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Design and conduct of the experiments, data analysis and final write up	65%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Rothnie C	Experimental work	
Spring DJ	Experimental work	
Verrier ED	Design of experiment	
Venardos K	Experimental work	
Kaye D	Design of experiment	
Phillips DJ	Design of experiment, data analysis, manuscript revision	
Hedger MP	Design of experiment, data analysis, manuscript revision	
Smith, JA	Design of experiment, data analysis, manuscript revision	

Candidate's signature [redacted] **3/3/2012**

Declaration by co-author [redacted]

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Monash Institute of Medical Research**

Rothnie, C

Spring DJ

Verrier, ED

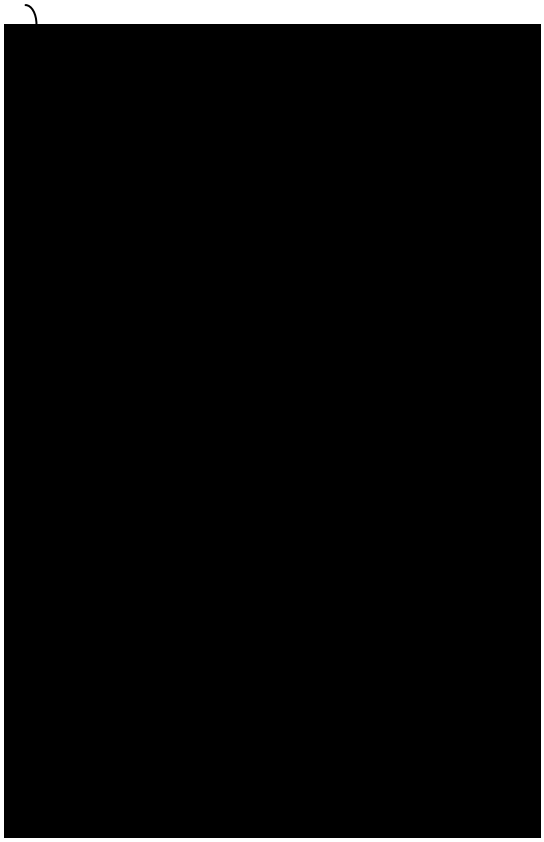
Venardos, K

Kaye, D

Phillips, DJ

Hedger, MP

Smith, JA



Regulation and actions of activin A and follistatin in myocardial ischaemia-reperfusion injury

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Abstract

Activin A, a member of the transforming growth factor-β superfamily, is stimulated early in inflammation via the Toll-like receptor (TLR) 4 signalling pathway, which is also activated in myocardial ischaemia-reperfusion (IR). Neutralising activin A by treatment with the activin-binding protein, follistatin, reduces inflammation and mortality in several disease models. This study assesses the regulation of activin A and follistatin in a murine myocardial IR model and determines whether exogenous follistatin treatment is protective against injury. Myocardial activin A and follistatin protein levels were elevated following 30 minutes of ischaemia and 2 hours of reperfusion in wild-type mice. Activin A, but not follistatin, gene expression was also up-regulated. Serum activin A did not change significantly, but serum follistatin decreased. These responses to IR were absent in TLR4$^{-/-}$ mice. Pre-treatment with follistatin significantly reduced the IR-induced myocardial infarction. In mouse neonatal cardiomyocyte cultures, activin A exacerbated, while follistatin reduced, cellular injury after 3 hours of hypoxia and 2 hours of reoxygenation. Neither activin A nor follistatin affected the hypoxia-reoxygenation (HR)-induced reactive oxygen species (ROS) production by these cells. However, activin A reduced cardiomyocyte mitochondrial membrane potential (MMP), and follistatin treatment ameliorated the effect of HR on cardiomyocyte MMP. Taken together, these data indicate that myocardial IR, through activation of TLR4 signalling, stimulates local production of activin A, which damages cardiomyocytes independently of increased ROS. Blocking activin action by exogenous follistatin reduces this damage.

Key words

Activin, follistatin, ischemia-reperfusion, heart

Introduction

During reperfusion of the ischaemic myocardium, cellular necrosis triggers a sterile inflammatory response through the release of endogenous molecules, designated damage-associated molecular patterns (DAMPs).[1] These molecules are sensed by receptors that are also involved in microbial pathogen recognition and inflammatory responses. One such group of receptors, which has been implicated in myocardial ischaemia-reperfusion (IR) injury, is the Toll-like receptors (TLRs).[2-3] TLR4, the receptor for the Gram-negative bacterial cell wall component, lipopolysaccharide (LPS), is expressed on cardiomyocytes and is responsible for LPS-induced myocardial dysfunction in endotoxaemia.[4-5] Activation of TLR4 has been demonstrated in myocardial IR, and pharmacological inhibition or genetic knock-out of TLR4 signalling has been shown to reduce myocardial IR injury in murine models.[6-7]

Activin A, a member of the transforming growth factor (TGF)-β superfamily of cytokines, is very widely-expressed and up-regulated in a broad range of inflammatory conditions.[8] It regulates the expression of key inflammatory mediators, such as interleukin-1 (IL-1), tumour necrosis factor (TNF) and nitric oxide *in vivo* and *in vitro.*[9] In animal models of LPS-induced endotoxaemia, activin A is one of the earliest cytokines to increase systemically following TLR4 activation.[10-11] However, $TLR4^{-/-}$ mice do not release activin A following LPS treatment.[11] In humans, acute myocardial infarction is associated with elevated serum activin A levels which correlate with peak serum creatine kinase concentrations.[12] In animal studies, activin A gene expression has been found to be up-regulated following myocardial IR

and it has also been implicated in myocardial remodelling in congestive heart failure.[13-14] As activation of TLR4 pathway may be one of the key features of IR-induced inflammation, these studies raise the possibility that activin A could be involved in the downstream inflammatory pathway.

Follistatin is a protein that binds activin A with high affinity and whose expression in can be induced by activin A and several other pro-inflammatory cytokines.[9] Following a LPS challenge, serum follistatin levels, like those of activin A, are markedly elevated.[15] Activin A-follistatin complexes are biologically inactive and bind to cell surface heparan sulphate-containing proteoglycans for internalisation and degradation.[16] Importantly, blocking activin A by administration of follistatin has been shown to considerably reduce inflammation and mortality in a mouse model of endotoxaemia.[11] Follistatin has also been shown to ameliorate injury in renal and hepatic IR injury.[17-18]

In the present study, we hypothesise that activin A expression is increased by myocardial IR and that follistatin may reduce myocardial injury through the binding of activin A.

Materials and Methods

Reagents

Bovine follistatin was purified in our laboratory from ovarian follicular fluid, as previously described.[19] Bovine, human and mouse follistatin have 97% identity at the amino acid level. Human recombinant activin A (100% amino acid identity with

mouse activin A; R&D systems, Minneapolis, MN, USA) was a generous gift from Dr. Craig Harrison (Prince Henry's Institute, Victoria, Australia).

Experimental animals

All animals were maintained in accordance with guidelines published by the National Institutes of Health and the Australian National Health and Medical Research Council. Experiments were approved by the Animal Ethics Committees of the University of Washington, Monash University and the Baker IDI Heart and Diabetes Institute.

In situ murine IR model

Wild-type C57BL/6J and C57BL/10ScNJ TLR4$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, Maine, USA) between 10-14 weeks old, 22-26 g body weight, were subjected to 30 minutes ischaemia followed by 120 minutes of reperfusion, as described previously.[6] Briefly, mice were anaesthetised with intraperitoneal pentobarbital sodium (100 µg/g body weight, Abbott Laboratories, North Chicago, Ill, USA), intubated and ventilated. A left parasternotomy was performed under a dissecting microscope (Zeiss, Oberkochen, Germany). A 7/0 silk suture (Tyco Health Care, Norwal, Conn, USA) was passed behind the left anterior descending artery (LAD), just distal to the left atrial appendage. The threads were then passed through the tip of a 22-gauge angiocatheter. The sutures could then be tightened and released by applying a clip at the end of the angiocatheter to restrict and restore blood flow. Sham mice underwent the same surgical procedures except the sutures were not tightened. In the treatment group, mice were injected intraperitoneally with 10 µg follistatin, diluted in 1 ml of 0.9% saline just after anaesthesia. Control mice received 1 ml of 0.9% saline alone.

Determination of area at risk (AAR) and infarct size

The AAR and infarct area in the left ventricle of the mice were determined, as previously described.[6] The LAD was re-occluded at the end of the experimental protocol and 4% Evans blue dye (Sigma Aldrich, St. Louis, MO, USA) was injected into the aortic root so that the LAD territory, which was the AAR, remained unstained as a result of the re-occlusion. Hearts were then removed, rinsed in 0.9% saline and embedded in 1% agarose gel (Invitrogen, Calsbad, CA, USA) in phosphate-buffered saline and sliced into 1 mm thick sections parallel to the short axis of the left ventricle and incubated in 1% triphenyltetrazolium choloride (Sigma Aldrich) at 37°C for 20 minutes and 10% formalin (Sigma Aldrich) for 24 hours. The slices were then weighed and photographed using a digital camera. The AAR and infarct area (area not stained by triphenyltetrazolium choride) were measured by using computer planimetry (ImageJ 1.21 software; National Institutes of Health, USA).

Activin A ELISA

The activin A homodimer was measured by ELISA, as previously described.[20] The assay uses a sandwich design employing a monoclonal antibody (E4) raised against a synthetic peptide corresponding to residues 82-114 of the mature activin βA subunit and has been validated for both mouse serum and culture media.[20] The mean sensitivity was 13 pg/ml. The mean intra- and inter-assay coefficients of variation (CV) of the assay were 6.9% and 9% respectively.

Follistatin radioimmunoassay (RIA)

Follistatin was measured by RIA as previously described.[21] The assay employs human recombinant follistatin (National Hormone and Pituitary Program) as both standard and tracer. The assay sensitivity was 2.7 ng/ml, and the intra- and inter-assay CV values were 6.4 and 10.2%, respectively.

Quantitative reverse transcriptase-PCR (QRT-PCR)

The expression of *Inhba*, the gene for the βA subunit of activin A, and *Fst*, the follistatin gene, were measured by QRT-PCR, as described previously.[22] Total RNA was extracted from the left ventricular portion of the hearts using Trizol (Invitrogen) according to the manufacturer's instructions. Genomic DNA was removed using a DNAfree kit (Ambion, Austin, TX, USA). cDNA was synthesized using the Superscript kit (Invitrogen). QRT–PCR was performed using a Biorad iQ5 system (BioRad, Hercules, CA, USA) with FastStart DNA Master SYBR-green system (Roche, Basel, Switzerland). Data were analysed using relative quantification, normalised against 18S mRNA as the house keeping gene and presented as fold change compared with control samples. Primers for mouse 18S mRNA were 5'-ACCGCAGCTAGGAATAATGGAA-3' (forward) and 5'-TCGGAACTACGACGGTATCTGA-3' (reverse); for *Inhba* (activin A) were 5'-TGGAGTGTGATGGCAAGGTC-3' (forward) and 5'-AGCCA CACTCCTCCAC AATC-3' (reverse) and for *Fst* (follistatin) 5'-CCACTTGTGTGGTGGATCAG-3' (forward) and 5'-AGCTTCCTTCATGGCACACT-3' (reverse).

Mouse neonatal ventricular cardiomyocyte (NVCM) isolation and culture

NVCMs were isolated using a well established technique.[23] Briefly, neonatal (1-2 day old) C57BL/6J mice were killed by decapitation and the ventricles dissected and

exposed to trypsin (Gibco Laboratories, Grand Island, NY, USA) treatment overnight at 4°C. Cardiomyocytes were dissociated by serial collagenase ((Worthington Biochemical Corp., Freehold, NJ, USA) digestion at 37°C. Cells were then cultured in Dulbecco's modified Eagle's media (Gibco Laboratories) supplemented with 10% foetal bovine serum and antibiotics (Invitrogen, Carlsbad, CA, USA). Cultures were serum-starved for 24 hours, prior to experiment. The NVCM cultures were subject to 3 hours of hypoxia in a humidified 95% N_2/5% CO_2 flushed hypoxic chamber incubated at 37°C, followed by 2 hours of reoxygenation at 37°C in 5% CO_2/air, as previously described.[23] Normoxic control cells were incubated at 37°C in 5% CO_2/air only. Activin A (50 ng/ml) or follistatin (100 ng/ml) were added to cardiomyocyte cultures just before experimentation under normoxic and hypoxia-reoxygenation (HR) conditions. Culture media were collected at the end of the culture conditions and stored at -20°C prior to assay.

Measurement of lactate dehydrogenase (LDH)

Release of LDH as a marker of cellular necrosis was measured in media collected from NVCMs, as previously described.[23] Briefly, 100µl of 1 mmol/L NADH (Sigma Aldrich) was added to 800 ml of assay mix containing 125 mmol/L NaH2PO4 (pH 7.5) and 1.25 mmol/L sodium pyruvate (Sigma Aldrich). The LDH present in the samples reduces pyruvate to lactate using NADH as the electron donor. In the process NADH is oxidized to NAD^+ and no longer absorbs light. LDH activity was assessed spectrophotometrically by measuring the rate of decrease in absorbance at 340 nm over 2 min.

Measurement of reactive oxygen species (ROS) production

ROS production was measured as previously described.[23] Briefly, ROS production was measured using the ROS sensitive flurochrome 2',7'-dichlorodihydro-fluorescein diacetate (H2DCF-DA, Invitrogen). Cardiomyocytes were incubated with phosphate-buffered saline supplemented with H2DCF-DA (10 μmol/L). Fluorescence measurements (excitation 485 nm, emission 520nm) were expressed as relative fluorescence units/second (RFU/sec), with background fluorescence subtracted from the average of 8 readings.

Measurement of mitochondrial membrane potential (MMP)

MMP was measured using the fluorescent probe 5, 5', 6, 6' -tetrachloro-1,1,3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Invitrogen) according to the manufacturer's recommendations, as previously described.[23] Inhibition of the electron transport chain promotes membrane depolarization which is detected by the JC-1 probe via a decrease in red/green fluorescence intensity ratio. A minimum of 5 replicates were measured for each of these parameters and expressed as percentage change to the normoxic control group.

Statistical analyses

The Graphpad Prism 5 graphical and statistics package (GraphPad Software Inc., San Diego, CA, USA) was used for presentation and analysis of the data. Data are expressed as mean ± standard error of the mean (SEM). Student's *t*-test and one way ANOVA were used to compare group data, as appropriate. Bonferroni's and Dunnett's post tests were used for multiple comparisons. A p value ≤ 0.05 was considered to be statistically significant.

Results

Myocardial activin A protein and mRNA levels following IR

After 30 minutes of ischaemia, using the *in situ* IR model, no changes were seen in myocardial or serum activin A protein levels between the ischaemia and sham surgery groups (data not shown). However, myocardial activin A protein levels were increased by 16% and mRNA expression rose over 6-fold after a further 2 hours of reperfusion compared with the sham surgery group ($p<0.05$, Fig. 1A, 1C). In contrast to the wild-type mice, neither activin A protein nor mRNA levels were significantly up-regulated in $TLR4^{-/-}$ mice following IR (Fig. 1B, 1D).

Myocardial follistatin protein and mRNA levels following IR

Myocardial and serum follistatin levels did not change after 30 minutes of ischaemia (data not shown). Myocardial follistatin protein levels increased by 31% after a further 2 hours of reperfusion. However, myocardial follistatin mRNA did not change significantly (Fig 2A, 2C). There were no changes in follistatin protein or gene expression after IR in $TLR4^{-/-}$ mice (Fig. 2B, 2D).

Serum activin A and follistatin levels following IR

There was no significant change in serum activin A levels following 30 minutes of ischemia and 2 hours of reperfusion. However, a 33% reduction in serum follistatin levels was observed (Fig 3A, 3C). In $TLR4^{-/-}$ mice, there were no changes in activin A and follistatin levels in serum following IR (Fig 3B, 3D).

Effect of follistatin pre-treatment on cardiac IR injury in vivo

In order to examine whether inhibition of activin A action by blockade with follistatin can reduce myocardial IR injury, mice were pre-treated with follistatin and then subjected to *in situ* myocardial IR. Pre-treatment with follistatin resulted in a significantly smaller (45% vs. 55%, $p<0.05$) myocardial AAR compared with the control group (Fig. 4A). However, the infarct area, expressed as a ratio of the AAR, was significantly smaller in the follistatin-treated group (22% vs. 38%, $p<0.05$; Fig. 4B).

Effect of follistatin on cardiomyocyte LDH release in vitro

In order to confirm the protective effects of follistatin seen *in vivo*, mouse NVCM cultures undergoing HR *in vitro* were treated with activin A or follistatin, with LDH levels measured as a marker of cellular necrosis. Addition of activin A increased LDH levels by 41% ($p<0.0001$ vs. untreated HR control), whereas follistatin treatment resulted in a 32% ($p<0.0001$) reduction in LDH levels compared with untreated HR controls (Fig. 5). These data indicate that activin A increases cell death in NVCM subjected to HR in culture, which can be blocked by follistatin.

Effect of activin and follistatin treatment on cardiomyocyte ROS production and MMP

The effects of activin A and potential protective mechanisms of follistatin in IR/HR were investigated by measuring cardiomyocyte ROS production and MMP. Activin A treatment, under normoxic conditions, did not result in a significant change in cardiomyocyte ROS production, but significantly reduced the MMP of these cells (Fig. 6A-B). As expected, HR resulted in 45% increase in ROS production and reduced MMP to 74%. Follistatin treatment under HR conditions did not reduce ROS

production but significantly restored MMP (74% to 91% of normoxic control; Fig. 7A-B)

Altogether, these data suggest that IR, acting through TLR4, stimulates activin A expression in the myocardium, which is able to damage the cardiomyocytes. Blocking activin action by exogenous follistatin reduces the subsequent damage. However, the damage to cardiomyocytes caused by activin A appears to be independent of, and hence is not mediated by, increased ROS production.

Discussion

In this study, we have demonstrated an increase in activin A and follistatin protein in the myocardium of wild-type mice, but not in TLR4$^{-/-}$ mice, during IR. The data suggest that the increase in activin A is due to increased expression in the myocardium, but the increase in follistatin may arise by increased uptake from the systemic circulation. The data also indicate that the TLR4 signalling pathway is an essential trigger for activin A release in myocardial IR. Blocking activin A activity using follistatin reduced myocardial infarct size *in vivo. In vitro*, cardiomyocyte injury following HR was significantly improved by follistatin treatment. These data suggest a causative role for activin A in myocardial IR injury and that blocking activin effects with follistatin can be protective in this setting (Fig. 8).

Similar IR injury-induced activin A gene expression has been documented in the kidneys, the brain and the heart.[17, 24-25] However, there have not been any previous studies which directly measured tissue activin A and the levels of follistatin protein. In this study, we observed that both activin A and follistatin protein are present within

the myocardium. This may represent activin A-follistatin complexes that are bound to cell surface heparan sulphate-containing proteoglycans and are biologically inactive. The activin A ELISA measures total activin A and cannot differentiate between free and bound form of the protein.[20] We have previously shown that activin A and follistatin proteins are detectable in all major organs of the body and this large pool of extracellular activin A-follistatin complexes is readily displaceable by heparin administration.[26-28] This high background level of activin A may partially obscure the magnitude of the increase in tissue free activin A levels following myocardial IR. However, the QRT-PCR data confirms that there is significant *de novo* synthesis of activin A in the myocardium following myocardial IR. In contrast, myocardial follistatin protein levels were elevated without any increase in gene expression, while serum follistatin levels actually decreased. This suggests that the increased myocardial follistatin levels are not due to *de novo* local synthesis, but may arise from the systemic follistatin pool. This provides a potential mechanism to explain how systemically-administered follistatin might enter the myocardium to target activin A and reduce IR injury.

The effect of LPS on activin A release is mediated by NFκB activation through MyD88-dependent TLR4 pathways.[11] TLR4 is widely expressed and cells capable of producing activin A are not limited to immune cells, but include epithelial cells, endothelial cells, cardiomyocytes, neurons and hepatocytes.[2] The current results indicate that TLR4 may be involved in the regulation of activin A in the myocardium as $TLR4^{-/-}$ mice did not show significant changes in activin A and follistatin gene and protein expressions compared with sham surgery controls.

In many cell systems, activin A has been identified as a pro-apoptotic factor.[29] Our findings are consistent with this role. Although *in vitro* activin A treatment had no effect on cardiomyocyte ROS production, it reduced MMP to a similar extent as that seen in HR. The effects of activin A *in vivo* is more complex and appears to be conditional on the type of organ studied and the experimental design. In the brain, activin A is known to be neuroprotective, while in the kidneys and the liver activin A has been found to be detrimental and the administration of follistatin was found to ameliorate IR injury in these organ systems.[17-18, 24] A study by Oshima and colleagues showed that that systemic over-expression of activin A was associated with reduced myocardial infarct size following IR.[14] However, in that study, serum follistatin was not measured in the activin A over-expressed mice. Since activin A is known to induce follistatin expression,[30] it is thus possible that follistatin levels may be elevated in activin A over-expressing mice and this may be responsible for the apparent protective effect of activin A in that study.

The therapeutic potential of follistatin as an anti-inflammatory, anti-fibrotic agent has been studied in a number of organ systems including the lungs and the liver.[31-32] The ability of follistatin to reduce IR-related injury has been demonstrated in the kidneys and, more recently, in the liver.[18] Our study extends the range of follistatin's protective effects to the myocardium. Unexpectedly, follistatin treatment *in vivo* reduced AAR. We do not believe this is due to technical error as a single experienced operator performed all the *in vivo* experiments and the mice were randomly assigned to either group. We postulate that follistatin may have potentially a hitherto unsuspected effect on collateral myocardial blood flow. Although a smaller AAR resulted in a smaller absolute infarct size in the follistatin treatment group, this

difference was adjusted for through the expression of infarct size as a percentage of AAR.

In support of the *in vivo* findings, follistatin treatment significantly reduced cardiomyocyte injury following HR *in vitro*. ROS production has been shown to be an important factor in initiating cell death through opening the mitochondrial permeability transition pores and decreasing MMP.[33] Decrease in MMP as a result of HR was almost completely restored following follistatin treatment. Follistatin does not signal through cell surface receptors. It's only known biological function is through the binding of TGF-β superfamily members, specifically activin A, myostatin and a number of bone morphogenetic protein (BMP) subfamily members including BMP4 and BMP7.[9] Although follistatin has the highest binding affinity for activin A, our study does not exclude the possibility that part of the protective effect of follistatin is through the inhibition of other members of the TGF-β superfamily such as BMP4, which has also been shown to be pro-apoptotic in the heart.[34] Further studies exploring follistatin as a therapeutic agent to limit the loss of MMP in the setting of IR are warranted.

Acknowledgements

We thank Sue Hayward, Wendy Winnall and Hui Wu for their technical support and expertise. The study was supported by a scholarship from the Royal Australasian College of Surgeons (to YC), grants from the National Health and Medical Research Council of Australia (to DJP and MPH), and the Victorian Government's Operational Infrastructure Support Program.

Statement of author contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; EV and DK contributed to the design of the studies; YC, CR, DS and KV conducted the experiments; and YC, DP, MH and JS wrote the manuscript.

Figure 1 Myocardial activin A levels in wild-type (WT) and TLR4$^{-/-}$ mice following ischaemia-reperfusion (IR).

Myocardial activin A protein levels in WT mice **(A)** and TLR4$^{-/-}$ mice **(B)** in IR and sham surgery groups measured by ELISA (N=5-6 mice in each group). Myocardial activin A mRNA levels in WT mice **(C)** and TLR4$^{-/-}$ mice **(D)** in IR and sham surgery groups measured by QRT-PCR. Activin A mRNA levels were normalized to 18S mRNA and expressed as fold increase over sham surgery group (N=3 mice in each group). Data are mean ± SEM; * p<0.05; ns, non significant.

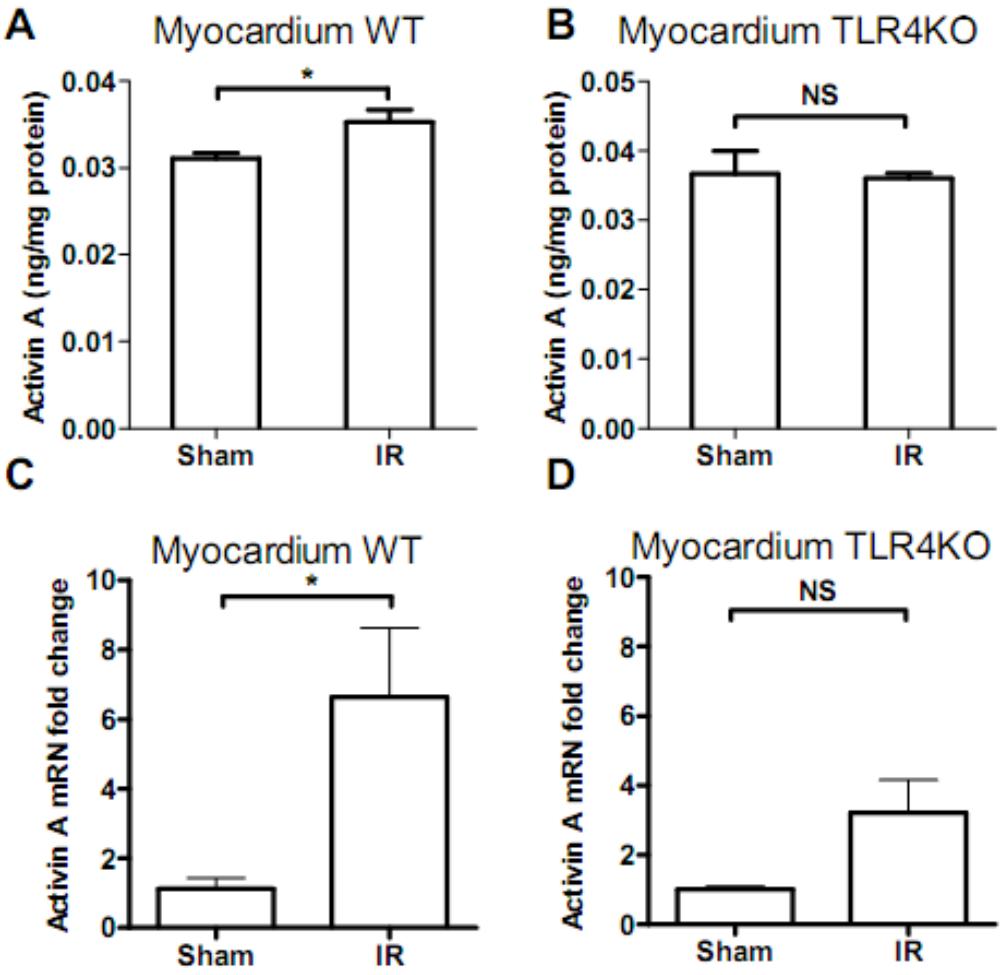


Figure 2 Myocardial follistatin levels in wild type (WT) and TLR4$^{-/-}$ mice following ischaemia-reperfusion (IR).

Myocardial follistatin protein levels in WT mice **(A)** and TLR4$^{-/-}$ mice **(B)** in IR and sham surgery groups as measured by RIA (N=5-6 mice in each group). Myocardial follistatin mRNA levels in WT mice **(C)** and TLR4$^{-/-}$ mice **(D)** in IR and sham surgery groups measured by QRT-PCR. Follistatin mRNA levels were normalized to 18S mRNA and expressed as fold increase over sham surgery group (N=3-4 mice in each group). Data are mean ± SEM; * p<0.05; ns, non significant.

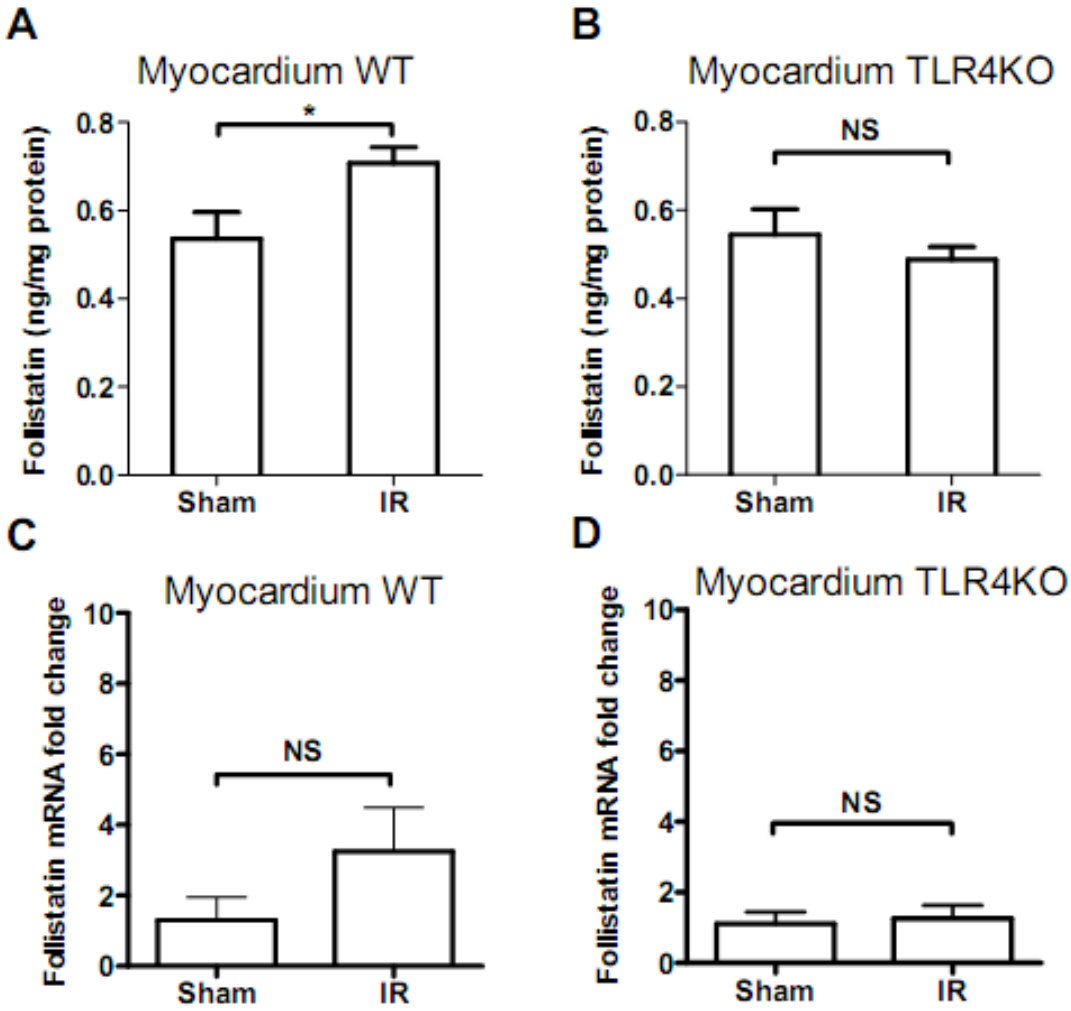


Figure 3 Serum activin A and follistatin protein levels following ischemia reperfusion (IR).

Serum activin A protein levels after IR in wild type (WT) mice **(A)** and TLR4$^{-/-}$ mice **(B)** measured by ELISA. Serum follistatin protein levels after IR in WT **(C)** and TLR4$^{-/-}$ mice **(D)** measured by RIA. N=5-6 mice in each group. Data are mean ± SEM; * $p<0.05$; ns, non significant.

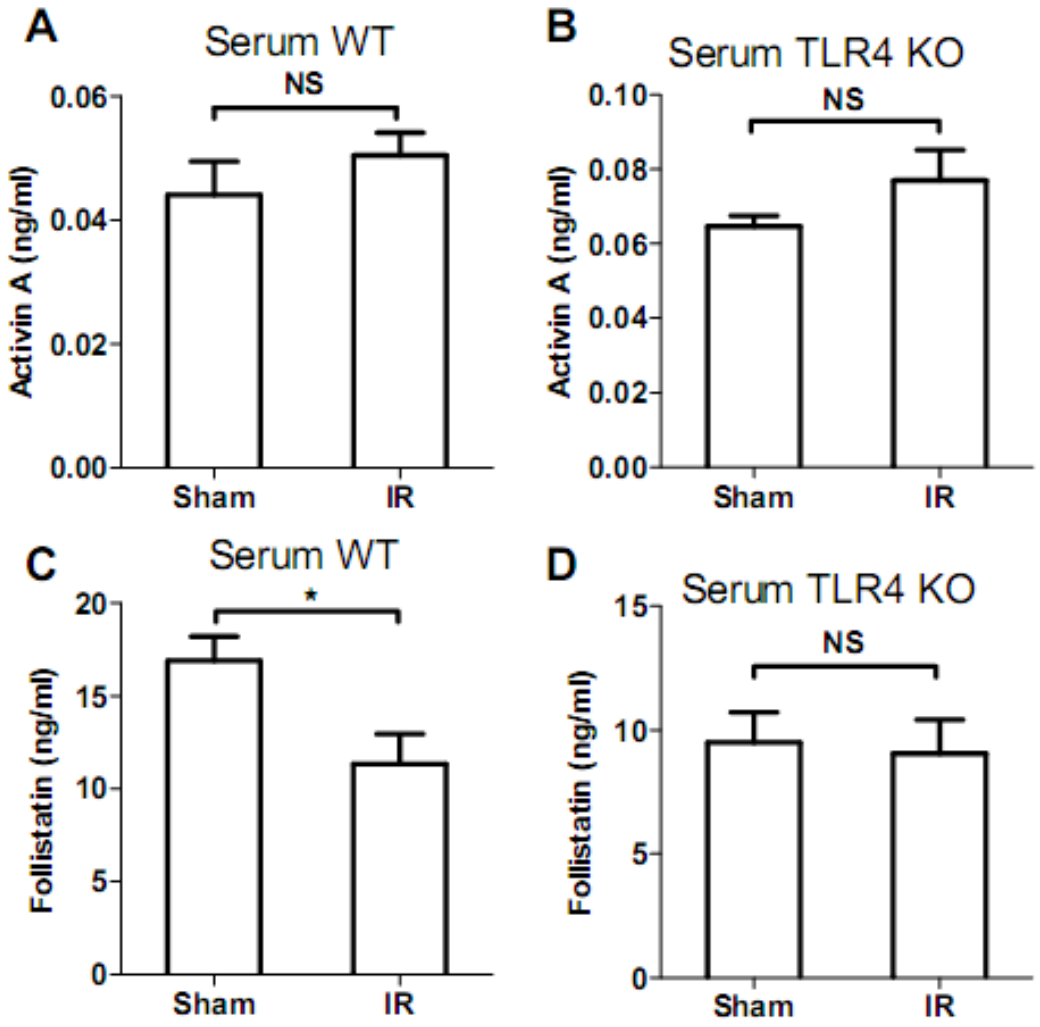


Figure 4 Effect of follistatin pre-treatment on myocardial infarct size following ischaemia-reperfusion.

(A) Area at risk of myocardium defined by Evans blue dye in saline control and follistatin treated groups. (B) Infarct size as a ratio of the area at risk in saline control and follistatin treated groups (N=9 mice in each group). Data are mean ± SEM; * $p<0.05$.

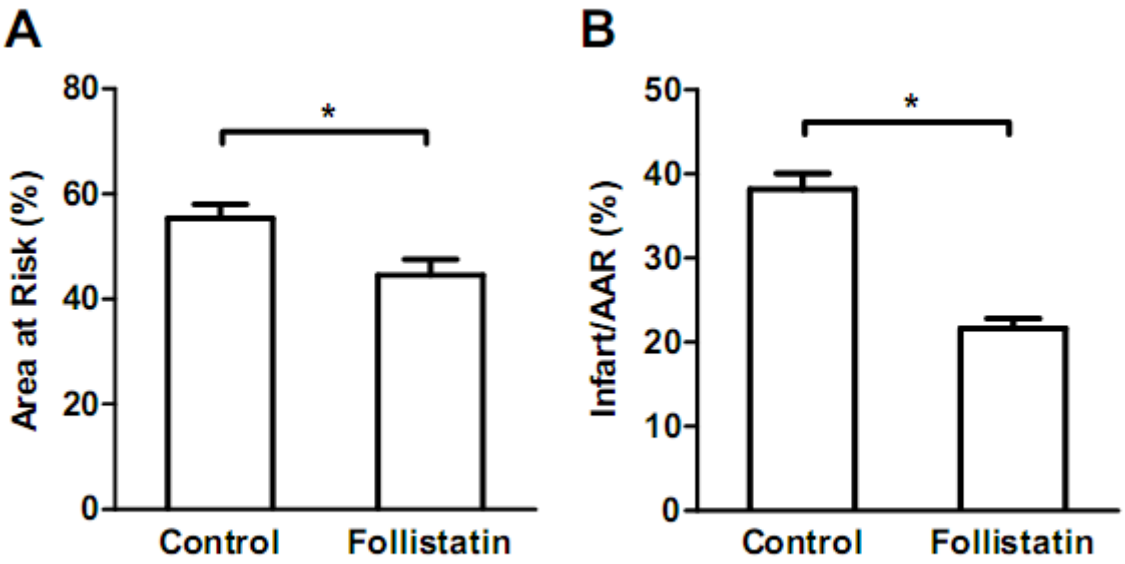


Figure 5 Effect of activin A and follistatin co-treatment on LDH release from cultured mouse NVCM undergoing hypoxia re-oxygenation (HR) *in vitro.*

LDH, a marker of cellular injury, was measured in the culture media in mouse neonatal cardiomyocyte culture in normoxic and hypoxia (3 hours) and re-oxygenation (2 hours) conditions with or without activin A or follistatin treatment. The level of LDH release from the HR group without any treatment was used as a reference group. Normoxic control and HR with the addition of either activin A or follistatin were expressed as percentage changes from the HR control. Data are mean ± SEM. Experiments were performed in triplicate in three separate experiments producing similar results. *** $p<0.001$

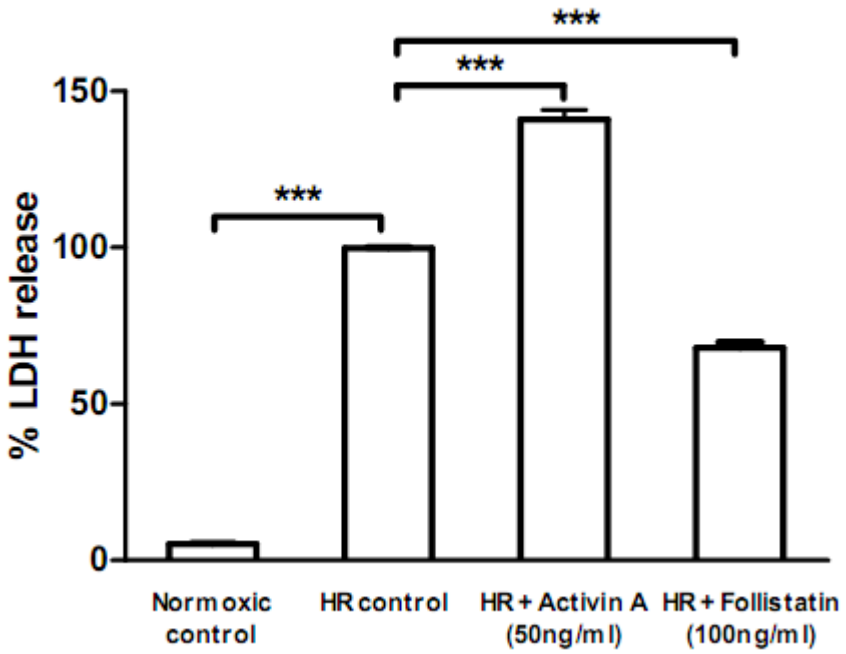


Figure 6 ROS production and mitochondrial membrane potential in cultured mouse NVCM following activin A treatment under normoxic conditions.

Changes in ROS production (A) and mitochondrial membrane potential (B) measured in 5 hour cultures under normoxic conditions with or without activin A co-treatment. Data are mean ± SEM. Experiments were performed with 6 replicates (data shown) and the experiment was repeated twice with similar results. *** $p<0.001$; NS, non significant.

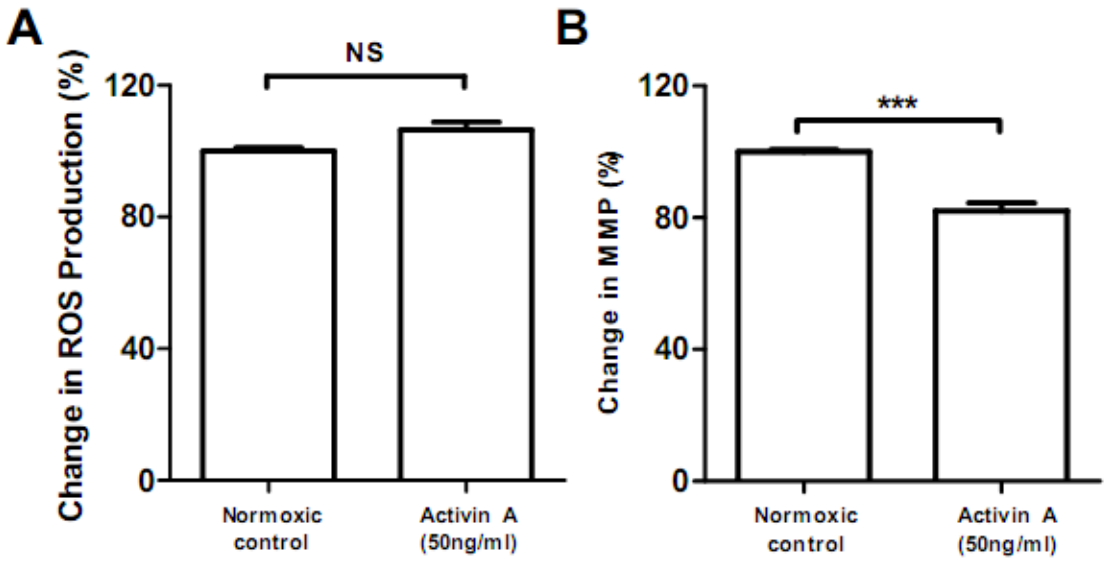


Figure 7 ROS production and mitochondrial membrane potential in cultured mouse NVCM following follistatin treatment in hypoxia-reoxygenation condition.

Changes in ROS production (A) and mitochondrial membrane potential (B) measured following 3 hours of hypoxia and 2 hours of re-oxygenation with or without follistatin co-treatment. Data are mean ± SEM. Experiments were performed in 6 replicates and the experiment was repeated twice with similar results. * p<0.05, ** p<0.01, *** p<0.001; NS, non significant.

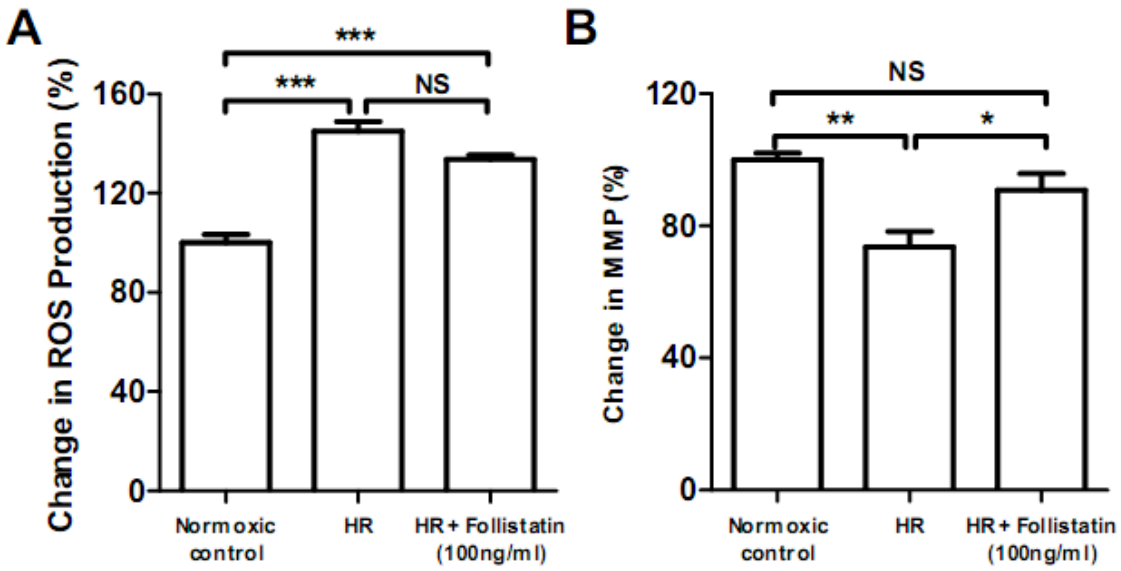
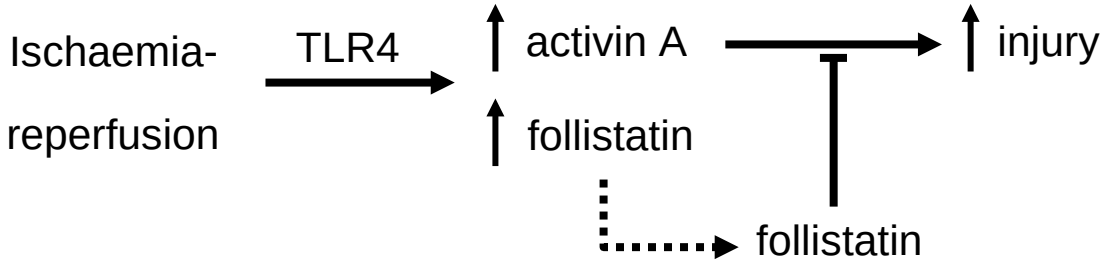


Figure 8 Summary diagram.

Ischaemia-reperfusion, acting via a TLR4-mediated mechanism, stimulates expression of activin A and its binding protein, follistatin, in cardiomyocytes. Administration of follistatin reduces the injury to cardiomyocytes caused by ischaemia-reperfusion or by hypoxia-reoxygenation *in vitro*. These data indicate that activin A is an intermediate in the pathway to cardiomyocyte damage, and that both endogenously-produced and exogenously-administered follistatin can act as a regulator of this pathway.



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CHAPTER 4

TUMOUR NECROSIS FACTOR-α STIMULATES HUMAN NEUTROPHILS TO RELEASE PREFORMED ACTIVIN A

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter Four

Declaration by candidate

In the case of Chapter Four, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Design and conduct of the experiments, data analysis and final write up	65%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Wu H	Experimental work	10%
Winnall WR	Experimental work	
Loveland KL	Design of experiment	
Makanji Y	Experimental work	
Phillips DJ	Design of experiment, data analysis, manuscript revision	
Smith JA	Design of experiment, data analysis, manuscript revision	
Hedger MP	Design of experiment, data analysis, manuscript revision	

Candidate's signature [redacted] **3/3/2013**

Declaration by co-authors

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(16) there are no other authors of the publication according to these criteria;
(17) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(18) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Monash Institute of Medical Research**
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Winnall, WR	
Loveland, KL	
Makanji Y	
Phillips, DJ	
Smith, JA	
Hedger, MP	

Immunology and Cell Biology (2011) 89, 889–896

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ORIGINAL ARTICLE

Tumour necrosis factor-α stimulates human neutrophils to release preformed activin A

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Activin A, a member of the transforming growth factor-β superfamily, is a critical early mediator of acute inflammation. Activin A release coincides with the release of tumour necrosis factor-α (TNF-α) in models of lipopolysaccharide (LPS)-induced inflammation. The source of circulating activin A during acute inflammation has not been identified and the potential contribution of leukocyte subsets was examined in the following study. Human leukocytes from healthy volunteers were fractionated using Ficoll gradients and cultured under serum-free conditions. Freshly isolated human neutrophils contained 20-fold more activin A than blood mononuclear cells as measured by enzyme-linked immunosorbent assay (ELISA), and both dimeric and monomeric forms of activin A were detected in these cells by western blotting. Activin A was predominantly immunolocalized in the neutrophil cytoplasm. Purified neutrophils secreted activin A in culture when stimulated by TNF-α, but were unable to respond to LPS directly. Although TNF-α stimulated activin A release from neutrophils within 1 h, activin subunit mRNA expression did not increase until 12 h of culture, and the amount of activin A released following TNF-α stimulation did not change between 1 and 12 h. Specific inhibition of the p38 MAP kinase signalling pathway blocked TNF-α-induced activin release, and the secretion of activin A was not due to TNF-α-induced neutrophil apoptosis. These data provide the first evidence that neutrophils are a significant source of mature, stored activin A. Stimulation of the release of neutrophil activin A by TNF-α may contribute to the early peak in circulating activin A levels during acute inflammation.
Immunology and Cell Biology (2011) **89,** 889–896; doi:10.1038/icb.2011.12; published online 29 March 2011

Keywords: cytokine; immunoassay; inflammation; leukocytes; lipopolysaccharide

Activin A, a member of the transforming growth factor-β superfamily of proteins, is a disulphide-linked homodimer of the inhibin βA subunits.[1] Recently, activin A has been established as a critical cytokine released early in endotoxaemia and other inflammatory syndromes.[2] Further, inhibiting activin A with follistatin, a high affinity activin-binding protein, results in decreased mortality in a mouse model of endotoxic shock.[3] Following a lipopolysaccharide (LPS) challenge, activin A is released into the circulation in a biphasic pattern.[3,4] The first peak occurs 1 h following LPS injection and coincides with the peak release of tumour necrosis factor (TNF)-α. This is suggestive of release of stored activin A rather than secretion from *de novo* synthesis, as induction of new activin A synthesis appears to require several hours to manifest following inflammatory stimuli in most cell types.[5–9] The source of this rapid increase in circulating activin A during acute inflammation has not yet been established.

One potential source of activin A during acute inflammation is the circulating leukocyte subsets. Monocytes, B lymphocytes and eosinophils are known to be capable of synthesizing activin A *in vitro*,[10–12] but none of these cell types have been shown to contain substantial stores of preformed activin A. In mice, activin A levels in the lungs increase significantly 1 h after an LPS challenge while there is a concurrent decrease in bone marrow activin A levels (our unpublished data). As systemic LPS induces a selective sequestration of circulating neutrophils into the lungs and mobilization of bone marrow neutrophils,[13,14] this suggests that these neutrophils could be a source of increased activin A levels in the lung following LPS administration. Neutrophils are the first line of host defense against infection, whereby activated neutrophils degranulate and release a large number of antimicrobial and pro-inflammatory proteins.[15] Neutrophils also express cell surface Toll-like receptors (TLRs)[15] and activation of TLRs stimulates activin A synthesis and secretion in diverse cell types.[2] These observations suggest that neutrophils may contain preformed activin A that is released during acute inflammation.

In the mouse model of endotoxaemia, the concurrent release of activin A and TNF-α raises the possibility of interplay between these two cytokines. Like TLR activation, TNF-α enhances activin A gene expression and protein secretion *in vitro* in a number of cell types including bone marrow stromal cell lines[16] and blood mononuclear cells.[12] Moreover, TNF-α is a potent neutrophil priming agent.[17] It upregulates adhesion molecules, augments the respiratory burst and is

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Received 15 October 2010; revised 16 January 2011; accepted 17 January 2011; published online 29 March 2011

also an important mediator of the apoptotic signalling pathways in neutrophils.[18] Furthermore, TNF-α activates mitogen-activated protein kinases (MAPKs) in neutrophils via TNF receptor-1, with differential regulation of ERK1/2, p38 and JNK pathways. Both the p38 and the JNK pathways have been implicated in the regulation of activin A synthesis in a mast cell line and in vaccinia virus-infected cell lines *in vitro*.[19,20]

In the present study, we provide evidence that neutrophils store mature activin A, localized predominantly in the cytoplasm, and that TNF-α rapidly induces its release via a p38 MAPK-dependent mechanism.

RESULTS

Activin A is rapidly released from LPS-treated whole blood and isolated total leukocytes *in vitro*, but not from isolated blood mononuclear cells

Human whole blood was examined for the release of activin A *in vitro* following LPS (50 ng ml^{-1}) stimulation. Activin A concentrations in LPS-treated blood after 4 h of culture were increased approximately twofold over basal levels of activin A (Figure 1A). Cycloheximide (a protein translation inhibitor) and actinomycin D (a RNA transcription inhibitor) had no effect on LPS-induced activin A release, indicating that LPS-induced activin A release from whole blood over 4 h did not require *de novo* mRNA or protein synthesis.

Isolated total blood leukocytes released both activin A (Figure 1B) and TNF-α (Figure 1C) in culture under basal conditions and when treated with LPS for 4 h. However, when neutrophils were removed and only blood mononuclear cells (comprising lymphocytes and monocytes) were cultured, LPS did not increase activin A in the culture medium over an equivalent period (Figure 1D).

Neutrophils, but not mononuclear leukocytes, contain preformed activin A

Neutrophils are the predominant leukocyte population in human blood. Freshly isolated neutrophils (purity >97%) were found to contain 20-fold higher activin A concentrations than the mononuclear cell population as determined by ELISA (Figure 2A). As the ELISA detects the dimeric form of activin A, this represents preformed, potentially biologically active activin A within neutrophils. Western blot analysis under non-reducing conditions revealed the presence of both dimeric and monomeric (βA subunit) activin A within the neutrophil cytoplasm (Figure 2B).

Using immunoctyochemistry, activin A was localized predominantly to the cytoplasm and perinuclear zone of neutrophils (Figure 2C). In contrast, mononuclear cells did not stain positively for the βA subunit, except for some monocytes, which had a faint, punctate staining pattern in the cytoplasm (Figure 2D). Given the extremely low level of activin A detected by ELISA (just above the threshold of detection) in these cells, neither monocytes nor lymphocytes are likely to contain significant amounts of the dimeric form of activin A under basal conditions. Taken together, these data indicate

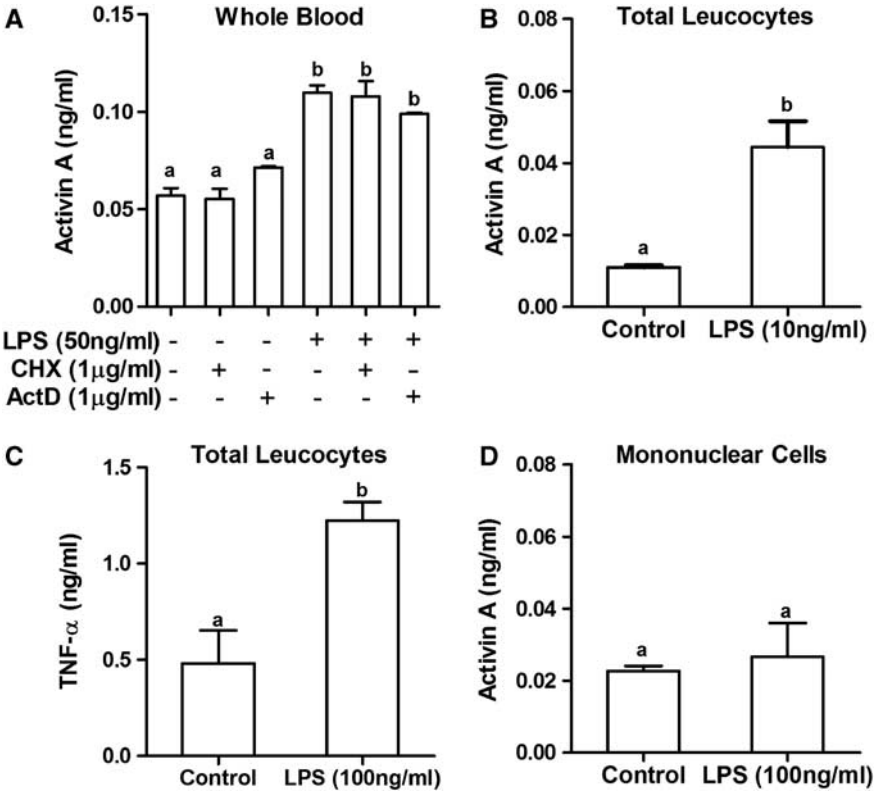

Figure 1 Activin A and TNF-α release from whole blood, isolated leukocytes and blood mononuclear cells *in vitro*. (**a**) Activin A secretion by human whole blood incubated with LPS and inhibitors of protein synthesis, cycloheximide (CHX) and actinomycin D (ActD) for 4 h. (**b**) Activin A secretion by isolated human total leukocytes treated with LPS for 4 h. (**c**) TNF-α secretion by isolated human total leukocytes treated with LPS for 4 h. (**d**) Isolated blood mononuclear cells treated with LPS for 4 h. Sera from whole blood and supernatants from cell cultures were collected and measured for activin A concentrations. Values with differing letter superscripts within each panel are significantly different at $P<0.05$. Data are mean ± s.e.m. Experiments were done in triplicate and representative data from three separate experiments are shown.

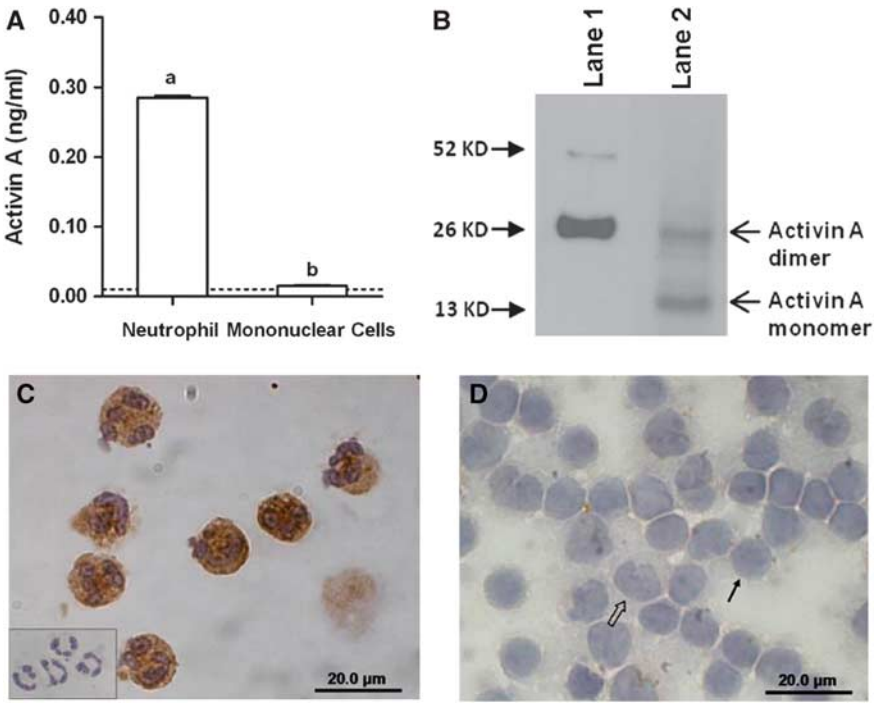

Figure 2 Activin A content in neutrophil and blood mononuclear cells and immunocytochemical localization of activin A. (**a**) Activin A concentration in neutrophils and blood mononuclear cell lysates. Two million neutrophils and blood mononuclear cells each were lysed in 200 μl of radioimmunoprecipitation buffer and activin A measured by ELISA. The dotted line represents the sensitivity limit of the assay. Values with a differing letter superscript indicate a significant difference at $P<0.05$. Data are mean ± s.e.m. Experiments were done in triplicate and representative data from three separate experiments are shown. (**b**) Western blot of neutrophil lysates under non-reducing conditions demonstrates the presence of both the dimeric and the monomeric forms of the activin A subunit within neutrophils. Lane 1 is human recombinant dimeric activin A. The 52-kDa band represents dimerized activin A dimer. Lane 2 is a neutrophil lysate sample. (**c**) Immunocytochemical localization of activin A in neutrophils. The activin βA subunit is stained brown. Inset is a negative control. (**d**) Immunocytochemical localization of activin A in mononuclear cells. The solid arrow indicates a lymphocyte and the open arrow a monocyte.

that neutrophils contain preformed, potentially biologically active activin A, which is largely absent in mononuclear blood leukocytes.

Release of activin A from isolated neutrophils is stimulated by TNF-α but not by LPS

Isolated neutrophils did not increase their basal secretion of activin A when stimulated with LPS at concentrations up to 1000 ng ml^{-1} and cultured over a 12-h period (Figures 3A and B). Furthermore, neutrophil intracellular activin A content was not increased by LPS (data not shown). This suggests that over the 12-h culture period, LPS itself was unable to increase the production of dimeric activin A within neutrophils.

Several other neutrophil activating agents, including interleukin (IL)-8 (10 ng ml^{-1}–10 μg ml^{-1}), phorbol 12-myristate 13-acetate (1 mM) and *N*-formyl-Met-Leu-Phe (1 μM), were likewise unable to elicit increased activin A release from neutrophils (data not shown). In contrast, TNF-α stimulated activin A release from neutrophils in a dose-dependent manner (Figure 3C). This release occurred within the first hour of stimulation and did not increase over time (Figure 3D). It was estimated that the released activin A represented ~40–50% of the stored cytoplasmic activin A.

Neutrophil activin A mRNA expression is upregulated by LPS

Treatment with TNF-α had no effect on activin A mRNA expression at 1 and 4 h of culture and had only a moderate (twofold) effect after 12 h in culture (Figure 4A). In contrast, LPS caused a significantly larger change (ninefold) in activin A gene expression within 4 h (Figure 4B). However, as reported above, this increase in mRNA was not associated with an increase in either secreted or intracellular dimeric activin A concentrations as measured by ELISA.

Inhibition of p38 MAPK signalling blocks TNF-α induced activin A release

Activin A release was abolished by treating neutrophils with the p38 MAPK inhibitor, SB203580 (Figure 5A). Inhibition of JNK (SP600125) had no effect on the release of activin A from neutrophils and inhibition of ERK1/2 kinase (PD98059) resulted in a small (15%), although statistically significant, reduction in activin A release from neutrophils (Figures 5A and B).

Activin A release is not due to autolysis of apoptotic neutrophils

The p38 pathway is involved in TNF-α-induced neutrophil apoptosis.[18] As soluble cytoplasmic proteins may be released as a result of apoptosis-induced autolysis, neutrophil apoptosis as a potential mechanism of activin A release was assessed. At both 1 and 4 h, TNF-α treatment resulted in a small increase in neutrophils entering apoptosis (annexin V^{high}, propidium iodide (PI)^{low} cells) compared with the untreated controls, but the number of disrupted cells in late apoptosis was very low (3–7% of total) in both untreated and treated groups at either of these time points (Figure 6). These data suggest that the release of activin A from neutrophils after stimulation with TNF-α was not due to increased cell disruption associated with neutrophil apoptosis.

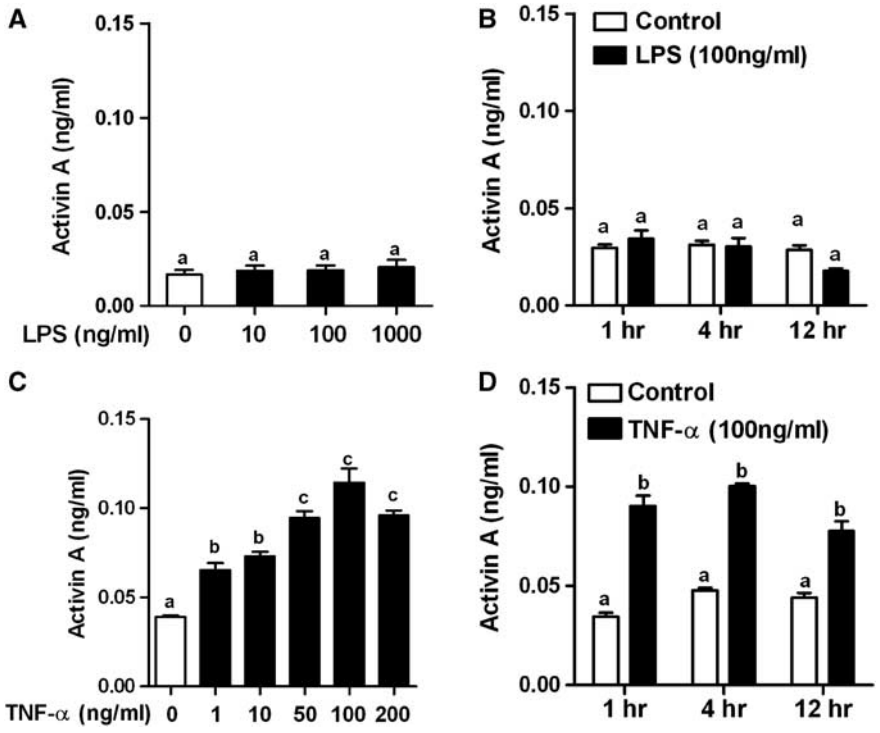

Figure 3 Dose-response and time-course studies of activin A release from neutrophils *in vitro*. (**a**) Neutrophils were cultured with increasing doses of LPS for 4 h and activin A concentrations were measured in the culture medium. (**b**) Neutrophils were cultured with or without 100 ng ml^{-1} LPS for 1, 4 and 12 h. (**c**) Neutrophils were cultured with increasing doses of TNF-α and activin A concentrations measured in the culture medium. (**d**) Neutrophils were cultured with or without 100 ng ml^{-1} TNF-α for 1, 4 and 12 h and activin A concentrations measured in the culture medium. Values with differing letter superscripts within each panel are significantly different at $P<0.05$. Data are mean ± s.e.m. Experiments were done in triplicate and representative data from three separate experiments are shown.

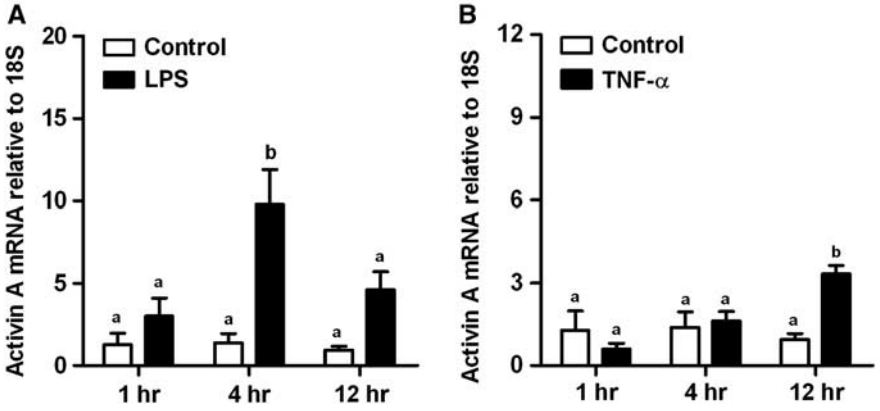

Figure 4 Measurement of activin A subunit mRNA expression in neutrophils by QRT-PCR. Activin A subunit mRNA expression following TNF-α (**a**) or LPS (**b**) challenge over a 12 h period. Activin A subunit mRNA levels were normalized to 18S RNA and expressed as fold increase over the 1-h neutrophil control sample. RNA was extracted from triplicate wells and QRT-PCR was performed in duplicate. Values with differing letter superscripts within each panel are significantly different at $P<0.05$. Data are mean ± s.e.m. of triplicate wells. Representative data from three separate experiments are shown.

DISCUSSION

This study has identified human neutrophils as an important source of mature, preformed activin A. Treatment of purified neutrophils with TNF-α caused a rapid increase in the release of activin A, which was not dependent on new protein synthesis, but rather on secretion of cytoplasmic stored activin A, through the activation of the p38 MAPK-dependent signalling pathway. These data suggest that neutrophils may be significant contributors to the early surge of activin A concentrations in the blood during acute inflammation, and that TNF-α is an important regulator of its release.

Interestingly, both monomeric and dimeric forms of activin A were present in purified neutrophil preparations. The monomeric form of activin A has been previously isolated from bovine follicular fluid.[21,22] It has reduced receptor binding affinity and biological activity compared with dimeric activin A. The origins and functions of the monomeric form of activin A remain to be determined. It should be

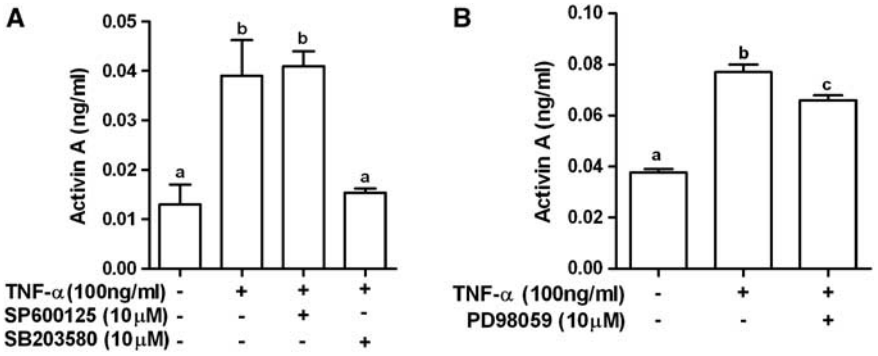

Figure 5 Effects of MAP kinase pathway inhibitors on TNF-α stimulated activin A release by neutrophils. Neutrophils were cultured for 4 h in the presence of TNF-α with or without inhibitors of the MAP kinase signalling pathways. (**a**) SP600125 inhibits JNK and SB203580 inhibits p38 MAP kinases, respectively. (**b**) PD98059 inhibits ERK1/2. Values with differing letter superscripts within each panel are significantly different at $P<0.05$. Data are mean ± s.e.m. Experiments were done in triplicate and representative data from three separate experiments are shown.

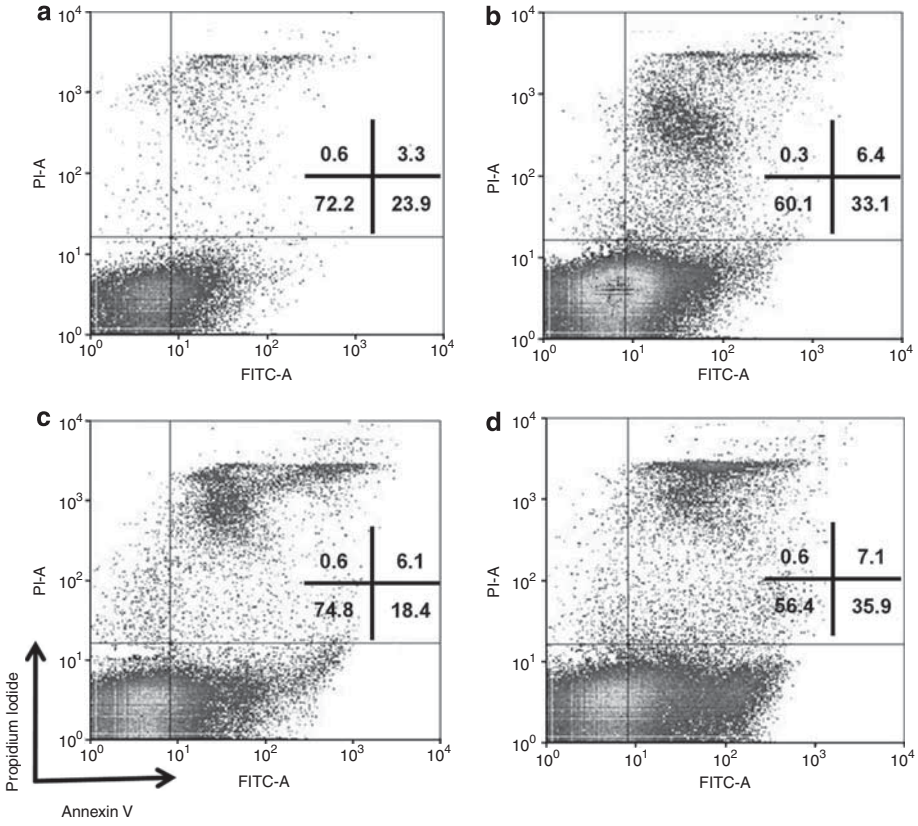

Figure 6 Flow cytometric analysis of neutrophil apoptosis in culture. Untreated and TNF-α-treated neutrophils collected after 1 and 4 h of culture were stained with PI and FITC-labelled annexin V. (**a**) Untreated neutrophils at 1 h. (**b**) TNF-α-treated neutrophils at 1 h. (**c**) Untreated neutrophils at 4 h. (**d**) TNF-α-treated neutrophils at 4 h. Insets represent the percentage of cells in each quadrant. Annexin V^{low}/PI^{low} (lower left) quadrant represents viable cells, annexin V^{high}/PI^{low} (lower right) quadrant represents early apoptotic cells and annexin V^{high}/PI^{high} (upper right) quadrant represents cells in late apoptosis with disrupted membranes.

noted that the ELISA cannot detect monomeric activin A, hence all measurements of activin A in culture medium and blood represent the dimeric, more biologically active, form of activin A.

Bacterial LPS is a potent stimulator of activin A synthesis and secretion in diverse cell types including monocytes, dendritic cells and vascular endothelial cells.[6,7,12] These effects are mediated through the MyD88-dependent signalling pathway linked to the TLR4 receptor. However, in contrast to its effects on whole blood and total leukocytes, LPS itself was unable to elicit activin A release from isolated neutrophils *in vitro* and only transiently increased activin gene expression after 4 h of culture. Moreover, although activin subunit mRNA was increased after LPS treatment, there was no corresponding increase in the level of intracellular or secreted activin A, as measured by ELISA. It is not yet clear why the increase in mRNA levels did not lead to increased activin A production at the later time points, but these observations clearly indicate that LPS does not directly regulate the acute release of stored activin A from neutrophils. In fact, neutrophils only express cell surface TLR4 at low levels[23] and do not respond to

LPS as effectively as other cell types. It has been suggested previously that contaminating monocytes are, in large part, responsible for the neutrophil response to LPS observed *in vitro*,[24] and this would be consistent with the results of the current study.

In contrast to the lack of effect of LPS, TNF-α caused a rapid release of dimeric activin A and a corresponding reduction in neutrophil intracellular activin A levels, and this action of TNF-α appears to be mediated via the p38 MAPK signalling pathway. In other studies, TNF-α stimulated activin subunit gene expression and *de novo* activin A synthesis in bone marrow stromal cells, fibroblasts, vascular endothelial cells and blood mononuclear cells.[12,25,26] However, the effects of TNF-α on activin subunit mRNA expression in human neutrophils was minor and only occurred after 12 h of stimulation. The intracellular signalling mechanism involved in TNF-α stimulated activin subunit mRNA expression has not been determined. It is not yet certain that the release of stored activin A from neutrophils and the regulation of activin subunit mRNA expression in a number of cell types involve the same TNF-α-mediated pathways.

Our *in vitro* data raise the possibility that TNF-α may be an important mediator of activin A release in acute inflammation *in vivo*. The data also suggest that in LPS-induced inflammation, TNF-α alone or possibly in conjunction with other inflammatory mediators, but not LPS itself, is responsible for stimulating neutrophils to release activin A. *In vitro*, the level of TNF-α produced by total blood leukocytes after stimulation with LPS was relatively small ($\sim 1.25\,ng\,ml^{-1}$), and would not be sufficient to stimulate maximal activin A release from neutrophils. The possibility must therefore be considered that other inflammatory mediators not examined in this study, in addition to TNF-α, could be capable of stimulating neutrophil activin A release. Moreover, circulating levels of TNF-α following LPS challenge *in vivo* are significantly higher than those observed *in vitro*,[3] and tissue-fixed macrophages and mast cells are rich sources of TNF-α that are absent from the *in vitro* setting, but would be present *in vivo*.[27,28] Consequently, further studies are required to investigate the precise link between TNF-α and activin A release in acute inflammation *in vivo*.

In all culture experiments, activin A was also secreted by untreated neutrophils. Previous studies have shown that neutrophils constitutively secrete cytokines such as IL-8 and IL-1β.[29,30] However, in the current experiments, the basal activin A levels at 1 and 12 h of culture were similar, indicating that the low levels of activin A in the untreated control wells are most likely due to spontaneous release from the isolated neutrophils at the time of plating, rather than continuous basal secretion during culture.

Neutrophil apoptosis is an important mechanism for limiting the extent of acute inflammation, and TNF-α induces neutrophil apoptosis via the p38 MAPK pathway.[18,31] Although it was confirmed in the present study that the release of activin A in response to TNF-α occurred independently of apoptosis-associated cell lysis, it may be significant that blocking p38 signalling pathway both reduces neutrophil apoptosis and abolishes activin A release. As activin A has been shown in many cell systems to be pro-apoptotic,[32–35] this raises the possibility that activin A may have an autocrine role in the apoptotic pathways in neutrophils. It is presently unknown whether neutrophils express activin A receptors.

There is an increasing appreciation of the immunomodulatory role that activin A has in the setting of acute and chronic inflammation. Activin A inhibits the production of pro-inflammatory cytokines such as IL-1β and TNF-α in activated monocytes/macrophages.[36,37] and antagonizes some of the downstream actions of IL-6 and IL-1β.[38,39] The identification of preformed mature activin A in neutrophils raises the possibility of a novel immunoregulatory role for the neutrophils involving activin A.[40] A recent study suggested that TLR activation in highly purified neutrophils induced the production of IL-10, an anti-inflammatory cytokine, rather than pro-inflammatory cytokines.[41] Stored activin A released by activated neutrophils in acute inflammation may therefore be part of the mechanisms involved in aiding the resolution of acute inflammation.

In conclusion, these data provide the first evidence that circulating neutrophils are a major site of stored activin A, and the release of activin A by these cells can be rapidly induced by the early inflammatory cytokine TNF-α acting via the p38 MAPK regulatory pathway. However, it remains to be determined whether neutrophil-derived activin A, stimulated by TNF-α, is the principal source of circulating activin A during acute inflammation *in vivo*. Several other cell types that might be involved directly or indirectly in response to inflammatory stimuli *in vivo*, such as tissue-fixed macrophages and bone marrow stromal cells, would not have been represented in these *in vitro* experiments, and further studies will be required to address this issue.

METHODS

Reagents

Ficoll-Paque Plus was purchased from Amersham Biosciences (Uppsala, Sweden). RPMI-1640, glutamax, penicillin/streptomycin antibiotics and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA, USA). Human recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). JNK inhibitor (SP600125) and p38 inhibitor (SB203580) were obtained from Merck (Darmstadt, Germany) and ERK1/2 inhibitor (PD98059) from Sigma Aldrich (St Louis, MO, USA). IL-8, phorbol 12-myristate 13-acetate, *N*-formyl-Met-Leu-Phe, cycloheximide, actinomycin D and phenol-extracted LPS (from *E. coli*, serotype 0111:B4) were also purchased from Sigma Aldrich. Antibodies to the activin (βA) subunit used for immunocytochemistry were a gift from Wylie Vale at the Salk Institute, La Jolla, CA, USA.[42]

White blood cell and neutrophil isolation and culture

Heparinized blood was collected from three healthy volunteers. For whole blood experiments, 1 ml of freshly collected blood was incubated without dilution at 37 °C and 5% CO_2 in 24-well culture plates. Unfractionated total leukocytes were obtained by removing red blood cells and platelets using dextran sedimentation. Serum was removed by centrifugation. Isolated leukocytes were cultured at a density of two million cells per well in 24-well plates in 1 ml of serum-free RPMI-1640 media supplemented with 1 mM glutamax, $50\,000\,U\,l^{-1}$ penicillin, $50\,mg\,l^{-1}$ streptomycin and non-essential amino acids. Neutrophils were isolated from blood using an established density-centrifugation method.[43] Purity of the isolated neutrophils was routinely >97% as determined by flow cytometry (Abbott Cell-Dyn 3200 flow cytometer, Abbott Park, IL, USA) and confirmed by manual counting of haematoxylin-stained neutrophil cytospin preparations. Neutrophils were cultured in 24-well plates at a density of two million cells per well, in 1 ml of serum-free supplemented RPMI-1640 media. The mononuclear blood cell fraction (purity >95%, consisting of 40–80% lymphocytes and 20–50% monocytes) was also recovered and cultured. Culture media were collected and centrifuged to remove residual cells. The supernatants were frozen at −20 °C until assayed. Cell pellets of neutrophils and mononuclear cells were lysed with radioimmunoprecipitation buffer[44] for measuring intracellular protein level and western blot analysis. All experiments were carried out in triplicate and repeated at least three times with blood drawn from different volunteers.

Western blot analysis of neutrophil and mononuclear cell lysates

Samples were separated by SDS-PAGE on 4–10% Tris-Tricine gels. The proteins were electro-transferred onto nitrocellulose membranes and the membranes blocked overnight at 4 °C in 5% bovine serum albumin (Sigma Aldrich). The membranes were probed with a mouse monoclonal anti-activin A antibody (E4) as previously described.[45] The membranes were then probed with an horseradish peroxidase-conjugated sheep anti-mouse antibody

(GE Healthcare, Little Chalfont, Buckinghamshire, UK). The protein bands were detected on ECL-film (GE Healthcare) using the Lumi-Light Western Blot substrate (Roche, Basel, Switzerland) as per the manufacturer's recommendation. Human recombinant activin A (dimeric form) (R&D Systems) was used as a positive control.

Activin A immunocytochemistry

Immunocytochemistry was performed on cytospin preparations of neutrophils and blood mononuclear cells, as previously described.[46] The only modification was the omission of an antigen retrieval step, which was not necessary on the cytospin preparations. The primary antibody used was a polyclonal rabbit antibody raised against residues 81–113 of the activin βA subunit.[42] Controls for non-specific binding of the secondary antibody were performed in all experiments by omitting the primary antibody and by the use of a class-matched isotype control.

Activin A and TNF-α ELISAs

Dimeric activin A was measured by ELISA, as previously described.[47] The assay uses a sandwich design employing a monoclonal antibody (E4) raised against a synthetic peptide corresponding to residues 82–114 of the mature activin βA subunit and has been validated for culture media. The mean sensitivity was 0.013 ng ml^{-1}. The mean intra- and inter-assay coefficients of variation of the assay were 6.9 and 9%, respectively.

TNF-α was measured using a Quantikine human TNF-α ELISA (R&D Systems), as per the manufacturer's instructions. The assay has a sensitivity of 0.0016 ng ml^{-1}.

Quantitative reverse transcriptase-PCR

Expression of INHBA, the gene that expressed the βA subunit of activin A was measured by quantitative reverse transcriptase-PCR (QRT-PCR), as described previously.[48] Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Genomic DNA was removed using a DNAfree kit (Ambion, Austin, TX, USA). cDNA was synthesized using the Superscript kit (Invitrogen). QRT-PCR was performed using a Biorad iQ5 system (BioRad, Hercules, CA, USA) with FastStart DNA Master SYBR-green system (Roche). Data were analysed using relative quantification, normalized against neutrophil 18S RNA as the housekeeping gene and presented as fold change compared with control samples. Primers for human 18S RNA were 5′-ACCGCAGCTAGG AATAATGGAA-3′ (forward) and 5′-TCGGAACTACGACGGTATCTGA-3′ (reverse) and for activin A were 5′-TGGAGTGTGATGGCAAGGTC-3′ (forward) and 5′-AGCCACACTCCTCCACAATC-3′ (reverse).[49]

Neutrophil apoptosis assay

Neutrophil apoptosis was detected by flow cytometry using FITC-labelled annexin V (BD Bioscience, Franklin Lakes, NJ, USA) and PI (Sigma Aldrich) using an established protocol.[50]

Statistical analyses

Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for graphing and statistical analyses. Data are expressed as mean ± s.e.m. Mann–Whitney, Kruskal–Wallis and two-way ANOVA were used to compare group data as appropriate. A P-value $\leqslant 0.05$ was considered statistically significant.

ACKNOWLEDGEMENTS

We would like to thank Sue Hayward for her technical support and Dr Elizabeth Williams for supplying the ERK1/2 inhibitor. The study is supported by a scholarship from the Royal Australasian College of Surgeons (to YC) and the National Health and Medical Research Council (NHMRC) of Australia. It was also supported in part by the Victorian Government's Operational Infrastructure Support Program.

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CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

In this thesis, a number of observations have been made regarding activin A and follistatin in CPB and myocardial IR injury. The key findings are that, first, activin A and follistatin are both released following CPB; second, follistatin is protective in myocardial IR injury; and third, neutrophils are a potential source of activin A in acute inflammation.

5.1 Activin A and follistatin release in CPB

Through the use of the sheep model of CPB, activin A was found to be released in a biphasic pattern in CPB, similar to that seen in LPS-induced systemic inflammation. This release is independent of the heparin effect that causes release of both bound activin and follistatin from tissue surfaces. The similarity in the release pattern in CPB and endotoxaemia suggests that the release may be also mediated by the TLR4 pathway. Other TLR agonists, such as Pam3Cys (TLR2) and PolyI:C (TLR3), may also release activin A, but lack the biphasic response that is seen in LPS-induced TLR4 activation (unpublished results from our laboratory). However, the magnitude of release by CPB is smaller than that induced by LPS. As has been previously shown, this LPS effect is entirely mediated by TLR4 signalling (Jones et al. 2007), and suggests that TLR4 may not be as robustly activated in CPB as is in endotoxaemia. There has been a number of papers that have shown elevated levels of HSPs following cardiac surgery (Becker et al. 2007; Szerafin et al. 2008) and these are thought to be endogenous ligands of the TLR4 signalling pathways (Chen and Nunez 2010). However, the ability of HSPs to activate TLRs has been called into question due to the possibility of *in vitro* contamination with LPS (Gao and Tsan 2003). Serum endotoxin levels were not measured in our study, as transmigration of gut bacterial products is known to occur in CPB and therefore may be a potential confounder.

Follistatin release in CPB was robust but did not occur while the sheep was on bypass. Its level peaked in the early post-operative period, coinciding with the second

peak of activin A release. This pattern was also observed in endotoxaemia (Jones et al. 2000; Jones et al. 2007). However, in control sheep undergoing sham CPB, the follistatin response to surgical trauma alone was also marked even though the changes in activin A levels were quite minimal. This is consistent with a previous study by Phillips and colleagues which demonstrated a comparable release of follistatin associated with surgical trauma caused by castration in male sheep (Phillips et al. 1996). From previous studies, activin A is known to induce follistatin release but our results also suggest that activin A may not be the only regulator of serum follistatin levels. It may be possible that the levels of follistatin following CPB are also under the control of other cytokines. IL-1β injection has been shown to cause elevated serum follistatin levels (Phillips et al. 1996) and soluble TNF-α receptor can reduce serum follistatin levels following LPS injection (Jones et al. 2004a). Furthermore, TLR3 activation can cause a robust release of follistatin with only a minor increase in circulating activin A levels (unpublished data from our laboratory). These data allow one to speculate that follistatin's role in acute inflammation may not be limited to activin A binding. It is also puzzling that, although the highest tissue levels of follistatin are found within the reproductive organs, it is released as part of the acute phase reaction and its levels correlate highly with serum IL-6 levels as demonstrated in our study. This suggests that follistatin may be an important modulator of acute inflammation. Serum IL-6 levels have shown correlations with clinical outcomes following cardiac surgery (Gueret et al. 2009; Mei et al. 2007; Musleh et al. 2009). It would be of interest to see if serum follistatin levels can provide additional predictive power over that of IL-6 regarding postoperative outcomes. Although heparin does cause a rapid and concurrent increase in activin A and follistatin levels during CPB (Chapter 2), the levels rapidly decline once the heparin dose is reversed with protamine at the end of CPB. By the 8^{th}-10^{th} postoperative hour, increases in serum follistatin levels are largely due to CPB *per se*

and not the heparin effect. It may therefore be possible to measure follistatin levels at that time point to study its correlations with clinical parameters

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One key question regarding activin A and follistatin which has not been addressed by our study is the functional significance of elevated activin A and follistatin levels in CPB. Although a large number of studies have examined the inflammatory response to CPB, interventions aimed at reducing the inflammatory response to improve clinical outcomes have been disappointing in clinical trials. Follistatin has shown great potential as an activin A neutralising agent in animal disease models (Aoki et al. 2005; Dohi et al. 2005; Patella et al. 2006), but without a better understanding of the biological effects of activin A and follistatin in CPB, it is unlikely to translate into clinical benefits.

5.2 Follistatin reduces myocardial IR injury

The expression pattern of activin A and follistatin was studied in a mouse model of myocardial IR injury. Both *in vivo* and *in vitro* data suggest that activin A is detrimental, whereas follistatin is protective, in myocardial IR. Although our study has not fully elucidated the mechanism of activin A's detrimental effect, *in vitro* data showing reduced mitochondrial membrane potential and increased LDH release following activin A treatment, suggest that it may be at least, in part, mediated through apoptosis. There are conflicting data regarding whether activin A is pro- or anti-apoptotic. One of the original descriptions of activin A was as a neuronal survival factor (Schubert et al. 1990). However, activin A has also been shown to cause plasma cell and hepatocyte apoptosis (Hully et al. 1994; Nishihara et al. 1993). The study by Oshima and colleagues demonstrated activin A is anit-apoptotic in cardiomyoctyes by up-regulation of Bcl-2 (Oshima et al. 2009). Indeed, even TGF-β, while it has been shown to be protective in myocardial IR, is also pro-apoptotic in some culture systems (Valderrama-Carvajal et al. 2002). It now appears that TGF-β

superfamily signalling is highly complex, being dependent on the cellular context and timing sensitive (Horbelt et al. 2012). In Oshima's study, the cardiomyocytes were pre-treated with activin A for eight hours before undergoing HR, whereas in our study, activin A was given at the time of the experiment. Possibly, prolonged incubation with activin A may prime the cells to be more resistant to HR but this may not be the case when activin A is given acutely at the time of HR. This difference in the duration of activin A exposure (*in vitro* through pre-treatment or *in vivo* through systemic over-expression) can potentially explain the apparent conflicting effects of activin A on cardiomyocyte survival. However, further studies are warranted to test these speculations

Another interesting finding in our study is that follistatin gene expression was not acutely elevated in the heart following myocardial IR. This is consistent with Oshima and colleagues' findings that follistatin gene in the heart was not elevated until three days after reperfusion (Oshima et al. 2009). However, the same study showed that, although follistatin gene expression was not elevated, FSRP was in fact rapidly elevated, around the same time as myocardial activin A gene upregulation. This is consistent with previous studies showing FSRP is preferentially distributed in the heart (Schneyer et al. 2001). FSRP also binds activin A but at slightly lower affinity than follistatin (Sidis et al. 2006). In the same study by Oshima and colleagues, FSRP gene knockout was associated with reduced infarct size following myocardial IR. This is hard to reconcile with our data showing a diametrically opposite effect of follistatin, a structurally closely-related protein. Once again, this may be due to the context and timing dependent effects of TGF-β superfamily members. Chronically reduced levels of FSRP may allow continued hyperactivity of activin A and other TGF-β superfamily members which may have distinct effects to acute exposure of these factors. Further studies are required to elucidate the mechanisms involved.

The expression of FSRP was not examined in our study as no protein assay is available.

5.3 Neutrophils as a source of preformed activin A

Through this study, results have implicated neutrophils as a potential source of preformed activin A in the body during acute inflammation. Activin A is widely distributed in the body. Preformed activin has been immunolocalised in a number of tissues including respiratory and gastrointestinal epithelia. However, the source of activin A in the LPS-induced inflammatory response remains elusive. Studies on the heparin effect have also demonstrated a large extracellular pool of activin A and follistatin, most likely in a complex (Jones et al. 2004b). It is not clear whether these sources of activin A are actually mobilised following inflammatory stimuli such as LPS or CPB. However, given the neutrophil's role in acute inflammation (Nathan 2006), it is highly likely that neutrophils may be an important source of activin A in acute inflammation.

Of the number of cytokines and neutrophil-activating agents tested, only TNF-α was shown to be able to consistently release activin A from neutrophils. Activin A and TNF-α serum levels increase almost concurrently in LPS-induced systemic inflammation (Jones et al. 2004a; Jones et al. 2007). It is therefore tempting to think that activin A release in that setting is due to TNF-α-induced activin A release from neutrophils. This hypothesis is yet to be proven. From published studies and data from our laboratory, neutrophils are probably not the only source of activin A in acute inflammation. Bone marrow stromal cells and new protein synthesis from tissues with high endogenous activin mRNA levels such as the liver may well also be involved (Wu et al. 2012, 2013).

It is intriguing to speculate the function of activin A in neutrophils. Studies on the function of activin A supports largely an immunoregulatory role (Hedger et al. 2011). Neutrophils are short-lived cells. After activation, their granules are released and they rapidly die by apoptosis. Neutrophil activating agents such as IL-8, phorbol 12-myristate 13-acetate and N-formyl-Met-Leu-Phe were unable to cause activin A release from neutrophils. This suggests that activin A release from neutrophils is not a passive phenomenon linked to degranulation but rather there are specific triggers for the release of activin A from neutrophils. In the study, results have also demonstrated that activin A release from neutrophils is not simply the result of autolysis. Considering the role of activin A in mediating apoptosis, it is an intriguing hypothesis that activin A is only secreted from neutrophils by specific apoptotic signals and once secreted, activin A acts in an autocrine fashion to cause neutrophil apoptosis.

It has also been recently shown that neutrophils may have a hitherto unappreciated immunoregulatory role in acute inflammation (Cassatella et al. 2009). Neutrophils are capable of secreting large amounts of IL-10 following bacterial infection, and neutrophil depletion in chronic infection promoted inflammation (Zhang et al. 2009b). The presence of activin A in neutrophils lends further support to the idea that neutrophils are not just phagocytic effector cells, but may also be involved in limiting acute inflammation through immunoregulatory cytokines such as IL-10 and activin A. Further studies on this aspect of neutrophil function are warranted.

Neutrophils are highly activated by both CPB and IR injury. Although it is conceivable that serum activin A levels are increased as a result of activated neutrophils, our study suggests that specific triggers (e.g. TNF-α) must be present for neutrophils to release activin A. LPS itself is unable to stimulate neutrophils to release activin and there were no significant differences in TNF-α levels between the CPB and the

surgical control group (Chapter 2). This suggests that, although neutrophils contain preformed activin A, it is probably not the source of elevated activin A serum levels in CPB. In our myocardial IR study, the reperfusion period is relatively short. Although neutrophil recruitment is an important feature of myocardial IR, it usually does not occur until about 6 to 12 hours after ischaemia. A two hour period of reperfusion may be too short for the neutrophils to accumulate within the myocardium and this makes it unlikely that infiltrating neutrophils are the primary source of elevated myocardial activin A. Based on these data, it appears that neutrophil-associated activin A may not play a significant role in increased serum and myocardial levels of activin A in CPB and myocardial IR in our study models. However, postoperative complications such as acute lung injury are associated with neutrophil activation, so that activin A may potentially be involved as it has been shown to cause an ARDS-like type of lung pathology (Apostolou et al. 2012).

5.4 Clinical implications of activin A and follistatin in cardiac surgery

Both large and small animal models have been used for the study of activin A in this thesis. Our studies have provided data to support the hypothesis that activin A and follistatin are expressed in CPB and myocardial IR. The various animal models used are well established and taken as a whole, do provide insights into the fundamental biology of activin A in acute sterile inflammation. The potential disadvantage of this approach is that the results may be influenced by differences in activin biology between the species and may not be directly translatable into clinical setting. Although there are considerable data to support a role for activin A and follistatin in infective and sterile inflammation, it is still not entirely clear how they are regulated and what functional significance they have. The fact that neutrophils contain preformed activin A is a novel finding that deserves further studies. The ultimate goal of researching into the basic biology of the inflammatory response in cardiac surgery is the hope that the basic understanding of the underlying immune processes will

lead to novel interventions or therapeutic options that will improve outcomes for patients. Follistatin has shown therapeutic potential in animal studies in limiting activin A associated inflammation and improving outcomes. It is hoped that these data presented here will serve as foundation for future research into activin A and follistatin in cardiac surgery related inflammation and this can one day translate to therapeutic applications of follistatin in limiting inflammation associated complications after cardiac surgery.

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