Regulation of Activins and Follistatin in Inflammatory and Metabolic Disorders

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Errata:

Chapter IV, Section 3.1, lines 3 and 4: activin A units should be pg/million cells presented as mean ± standard deviation

Chapter IV, Fig.1 legend: Panel C shows total activin A in the bone marrow neutrophil cultures

Copyright Notices

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List of abbreviations

8-Br-cAMP	8-bromo-cyclic AMP
ActRI	type I activin receptor
ActRII	type II activin receptor
ALK	activin receptor like kinase
AP-1	activator protein-1
BAMBI	bone morphogenetic protein and activin membrane-bound inhibitor
BMNPs	murine bone marrow-derived neutrophil precursors
Co-Smad	common partner Smad
CREB	cAMP-responsive element binding protein
CRP	C-reactive protein
CSF	cerebrospinal fluid
DAMPs	damage associated molecular patterns
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular single regulated kinase
ESR	erythrocyte sedimentation rate
FAST-1	fork-head activin signal transducer -1
FFA	free fatty acid
FS288	follistatin 288
FS315	follistatin 315
FSH	follicle-stimulating hormone
FSRP	follistatin-related protein
GLUT	glucose transporters
GM-CSF	granulocyte-monocyte colony stimulating factor
HbA1c	glycated haemoglobin

HDL	high density lipoprotein
HOMA-IR	homeostasis model assessment of insulin resistance
HSPG	heparan sulphate proteoglycan
IFN	interferon
IKK-β	IκB kinase-β
IL	interleukin
IL-1R	interleukin-1 receptor
IRAK	interleukin-1 receptor-associated kinase
IRS	insulin receptor substrate
I-Smads	inhibitory Smads
JNK	c-jun N-terminal kinase
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAL	MyD88-adaptor-like protein
MAP kinase	microtubule-associated protein kinase
MCP	monocyte chemoattractant protein
MyD88	myeloid differentiation primary response protein 88
NEMO	nuclear factor-κB essential modulator
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
PAMPs	pathogen-associated molecular patterns
PAI-1	plasminogen activator inhibitor-1
PCOS	polycystic ovary syndrome
PI3K	phosphatidylinositol 3-kinase

PKA	protein kinase A
PKC	protein kinase C
PMN	polymorphonuclear cells
RIA	radioimmunoassay
R-Smad	regulated Smads
SARA	Smad anchor for receptor activation
SBE	Smad binding element
sCD14	soluble CD14
Smad	mothers against decapentaplegic homologue
T2D	Type 2 diabetes mellitus
TAK-1	transforming growth factor -β activated kinase-1
TGF-β	transforming growth factor-β
Th1 cell	type 1 helper T cell
Th2 cell	type 2 helper T cell
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL-1 receptor adaptor protein
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
TPA	tetradecanoylphorbol 13-acetate
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter- inducing interferon-β

General Declaration

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 three published manuscripts. The core theme and title of this thesis is "Regulation of Activins and Follistatin in Inflammatory and Metabolic Disorders". The ideas, development and writing up of all the work in the thesis were the primary responsibility of the candidate, working within the Centre of Reproduction and Development at the Monash Institute of Medical Research under the supervision of Associate Professor Mark Hedger and Associate Professor David Phillips.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. Some activin, follistatin and tumour necrosis factor-α protein assays were performed by Susan Hayward. Collection of tissues from male mice and the injection of drugs or normal saline to male mice (Chapter II) were carried out with the assistance of Associate Professor Hedger, Associate Professor Phillips, Dr. Wendy Winnall, Dr. Yi Chen and Dr. Bernadette Scott. The immunohistochemical studies described in Chapter II were performed with the assistance of Dr. Yi Chen and Sarah Badelow. Patients' recruitment and blood sample collection (Chapter III) were performed by Dr. Michael Wu. Blood collection, bone marrow collection and cell

culture in Chapter IV were performed with the help of Dr. Yi Chen. Additional research advice, training and assistance were provided by Dr. Wendy Winnall, Kim Sebire and Lynda Foulds.

In the case of chapter II-IV, the candidates' contribution to the work involved the following:

Thesis chapter	Title	Status	Nature and extent of candidate's contribution
II	Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice.	Published	All laboratory work, except as indicated above. Design of experiments and research planning, analysis of data, drafts of the manuscript and preparation of figures for publication.
III	Correlation between blood activin levels and clinical parameters of type 2 diabetes.	Published	All laboratory work, except as indicated above. Analysis of data, drafts of the manuscript and preparation of figures for publication.
IV	Regulation of activin A release from murine bone marrow-derived neutrophil precursors by tumour necrosis factor-α and insulin.	Published	All laboratory work, except as indicated above. Design of experiments and research planning, analysis of data, drafts of the manuscript and preparation of figures for publication.

In addition, the candidate collaborated upon and contributed to the following publications related to this research:

Chen Y, Phillips DJ, McMillan J, Bedford P, Goldstein J, **Wu H**, Hedger MP & Smith JA 2011 Pattern of activin A and follistatin release in a sheep model of cardiopulmonary bypass. *Cytokine* **54** 154-160.

Chen Y, **Wu H**, Winnall WR, Loveland KL, Makanji Y, Phillips DJ, Smith JA & Hedger MP

2011 Tumour necrosis factor-alpha stimulates human neutrophils to release preformed activin A. *Immunol Cell Biol* **89** 889-896.

Winnall WR, **Wu H**, Sarraj MA, Rogers PA, de Kretser DM, Girling JE & Hedger MP 2012 Expression patterns of activin, inhibin and follistatin variants in the adult male mouse reproductive tract suggest important roles in the epididymis and vas deferens. *Reprod Fertil Dev* doi: 10.1071/RD11287. (Epub ahead of print)

Signed:

Date:

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Abstract

Activin A, a member of the transforming growth factor-β superfamily (TGF-β), increases in the circulation within one hour after administration of bacterial lipopolysaccharide. The source and regulation of activin A and follistatin, activin binding protein, during inflammation are poorly understood.

This thesis examined the major tissue sources and regulation of activin A and follistatin following lipopolysaccharide in adult male mice (Chapter II). All tissues examined contained activin A protein in untreated mice, but mRNA expression varied more than 100-fold across the tissues. The results indicated that the rapid increase in circulating activin A during lipopolysaccharide-induced inflammation was regulated at the post-mRNA level, apparently from newly-translated and stored protein. The bone marrow was implicated as the most significant source of pre-formed activin A protein, although the liver expressed the highest levels of activin β_A mRNA. Neutrophil precursors were the major cells containing significant activin A protein in the bone marrow. The lung was the only tissue with increased activin A protein, one hour post-lipopolysaccharide. Follistatin mRNA and protein were present in all tissues, with highest expression in the vas deferens.

Inflammation has increasingly been implicated in the development of type 2 diabetes (T2D), a metabolic disorder characterized by insulin resistance and hyperglycaemia. Both activin A and activin B, another member of the TGF-β superfamily, play important roles in glucose metabolism. In order to evaluate the potential roles of activin A, B and follistatin in T2D, this thesis examined circulating levels of these proteins (Chapter III). Serum activin A, B and follistatin levels were not different between subjects with a

normal oral glucose tolerance test, impaired glucose tolerance and/or impaired fasting glucose, or T2D. However, elevated levels of activin A and/or B were positively correlated with functional parameters of insulin resistance and T2D, specifically fasting glucose, fasting insulin, glycated haemoglobin and homeostasis model assessment of insulin resistance. These results suggested that serum activin A, B or follistatin were not independent risk indicators for T2D, but serum activin A and B levels were increased in parallel with increasing severity of disease in T2D patients.

Finally, as the bone marrow and the neutrophils, in particular, had been identified as a significant source of activin A protein during inflammation following lipopolysaccharide, neutrophils extracted from mouse bone marrow were examined to investigate the regulation of the release of activin A (Chapter IV). The findings showed that activin A protein levels in untreated neutrophil precursors was at least 7-fold higher than that in mononuclear cells. Lipopolysaccharide was not able to stimulate the release of activin A protein from cultured neutrophil precursors, but tumour necrosis factor-α (TNF-α), induced release of activin A within one hour. Pre-treatment of the neutrophil precursors with insulin ablated the response to TNF-α. However, TNF-α does not appear to be the only stimulator of activin A during inflammation, since lipopolysaccharide induced activin A release into the circulation of TNF-α null mice.

These data clarify the major sources of activin A release and the regulation of activin A release during inflammation. The discovery of the neutrophils' role in activin A release and the role of activins and follistatin in T2D provide new insight on the potential for follistatin to act as a specific therapeutic agent or neutrophils as a specific target cells for controlling activins' actions in various inflammatory diseases, and their consequences.

Chapter I – Literature Review, Aims and Hypotheses

I.1 General Introduction

Inflammation is the immune systems response to harmful stimuli (Shulman et al. 1990). Inflammatory disorders encompass a variety of pathologies that include septicaemia and acute infections such as meningitis, or more chronic inflammatory conditions, such as rheumatoid arthritis and inflammatory bowel disease. Furthermore, inflammatory components are implicated in several metabolic disorders, such as type 2 diabetes (T2D) and hypercholesterolemia (Guest et al. 2008; Jawień 2008).

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, has been found to be elevated in many inflammatory disorders, including septicaemia, meningitis, rheumatoid arthritis and asthma (El-Gendi et al. 2010; Kariyawasam et al. 2009; Phillips et al. 2001; Wilms et al. 2010). Lipopolysaccharide (LPS) is a component of the cell wall of gram negative bacteria which, in animal models such as rodents and sheep, is used to induce acute inflammatory conditions. It has been reported that in rats, mice and sheep the injection of LPS induces the release of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), accompanied by increased temperature and circulating leukocytes, and therefore, features similar to the clinic profile of acute inflammation (Jones et al. 2000; Jones et al. 2004a; Kimura et al. 1994; Klein et al. 1996a; Lamping et al. 1998; McAdam et al. 2000). After injection of LPS into the jugular vein of sheep, activin A release into the circulation was a very early event in the inflammatory cascade, preceding the release of TNF-α, which is one of the first released inflammatory factors in acute inflammation (Jones et al. 2004a). Although activin A is present in many tissues, it has not been known whether this release of activin A protein during inflammatory processes is from new production or stored protein and which

tissues are the major sources. Previous studies have shown that activin A is produced by a number of immune and non-immune cell types, including bone marrow stromal cells, epithelial and endothelial cells, fibroblasts and monocyte/macrophages (Hedger et al. 2011).

Inflammatory responses involve changes in the vascular system, immune system and various locally-responsive cells. Toll-like receptors (TLRs), signalling proteins that can stimulate release of activin A in innate and adaptive immune reactions (Phillips et al. 2009), have been found to be expressed in immune cells (neutrophils, monocytes, macrophages and dendritic cells) as well as non-immune cells (epithelial, endothelial and fibroblasts) (Delneste et al. 2007; Parker et al. 2005). Neutrophils are a critical immune cell type in the inflammatory response and numbers of neutrophils increase in acute bacterial infection or chronic inflammatory settings as a first line host defence (Normark et al. 2001). During acute inflammation, neutrophil numbers are elevated in the circulation and other tissues such as lung; further, anti-inflammatory agents reduce the infiltration of neutrophils into the lung (Zhang et al. 2012). Neutrophils are derived from the bone marrow, which is a major source of activin A, but production of activin A by these specific cells has not been studied previously.

An emerging major global health risk is T2D, a disease characterised by raised insulin, hyperglycaemia and elevated expression of pro-inflammatory cytokines, including TNF-α (Sjöholm and Nyström 2006). As in other developed countries, T2D in Australia is one of the commonest reasons for renal failure (Lopes 2009). Inflammation has increasingly been implicated as an important aetiological factor in the development of this condition. This conclusion is drawn from associations between elevated acute phase inflammatory

markers, such as TNF-α and C-reactive protein (CRP), and the development of T2D, especially for insulin resistance (Guest et al. 2008). Moreover, in mice models, serum levels of activin A are increased by TLR4 activation (Phillips et al. 2009). This is relevant, because both *in vitro* and *in vivo* studies have shown that TLR4 signalling is involved in the development of insulin resistance (Shi et al. 2006). It has been demonstrated that insulin treatment is able to attenuate systemic inflammatory responses and modulate monocyte/macrophage and neutrophil function (Cuschieri et al. 2008; Walrand et al. 2006). Notably, insulin can bind to receptors on the surface of human neutrophils and attenuate functions such as chemotaxis and phagocytosis (Safronova et al. 2001; Walrand et al. 2006). These studies suggest that elevated insulin and glucose during T2D could play a role in regulating neutrophil function and their potential production of activin A in this metabolic disease, which also appears to be chronic inflammatory disease. Since T2D is a metabolic disorder which has some inflammatory characteristics (Guest et al. 2008; Jawień 2008) and activin A has important effects in inflammatory disorders, it is worthwhile studying the links between activin A and T2D.

In this review, the following issues will be discussed: the origin and regulation of activin and its binding protein, follistatin, under normal conditions and during inflammation, the emerging relationship of activin and follistatin with T2D, and the role of the neutrophils in acute inflammation and metabolic disorders.

I.2 The Activins and Related Proteins

The TGF-β superfamily

Activins are members of the TGF-β superfamily, which encompasses regulators of diverse basic cellular biological processes, including nearly all stages of cellular growth and development (Herpin et al. 2004). The TGF-β superfamily is a large group of

structurally related homo-/hetero-dimeric proteins being joined by a single intrachain disulphide bridge and conserved cysteine residues (Daopin et al. 1992), which have been divided into subfamilies based on similar structural and signalling properties. These may be denoted as the TGF-β subfamily, the activin/inhibin subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Müllerian inhibiting substance) subfamily (Gordon and Blobe 2008; Herpin et al. 2004; Kingsley 1994; Shi and Massagué 2003).

The signalling of the members of each TGF-β superfamily starts with cell membrane heteromeric complexes receptors, comprising type I and type II serine/threonine-specific protein kinase receptors (ten Dijke et al. 1996). Both type I and type II receptors have three component parts: an N-terminal extracellular domain for binding, a transmembrane region and a C-terminal serine/threonine kinase domain (Shi and Massagué 2003). Type II receptors can bind ligands alone while type I receptors are recruited to bind ligands by the complex of ligand and type II receptors. Although type II receptors can bind with ligands alone, type I receptors are needed to trigger intracellular signalling. After binding with their ligands, type II receptors recruit and activate the type I receptors by phosphorylating a wedge-shaped GS region (TTSGSGSG sequence of the cytoplasm), which is located next to the kinase domain (Huse et al. 2001; Shi and Massagué 2003). The phosphorylated type I receptors activate specific receptor substrates (Smads), a family of transcription factors, and these Smad molecules form a complex moving into the cell nucleus to regulate gene transcription (Massagué and Wotton 2000). Specific details of activin A signalling will be discussed in more detail later in this review.

The activin/inhibin family: nomenclature and structure

Inhibin, another TGF-β family member which has nearly opposite function to activins, the activins themselves and follistatin, the activin binding protein (Gumienny and Padgett 2002; Ueno et al. 1987), were identified firstly as regulators of reproductive hormones released by the gonad to regulate the anterior pituitary hormone, follicle-stimulating hormone (FSH). The naming of activin and inhibin was based on their effects of

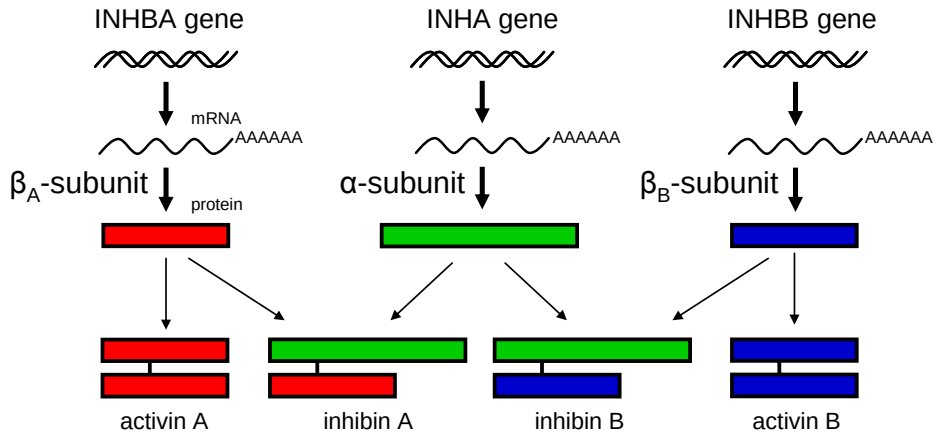

Figure 1 Homo- or heterodimers of the inhibin/activin family. The β_A subunit encoded by the INHBA gene and β_B subunit encoded by the INHBB gene can form homodimers of activin A and activin B, although the activin AB heterodimer also exists. The biological roles of activin A and activin B overlap although there is evidence showing they have some different biological activities in different tissues. The activin β_C subunit can form heterodimers with activin β_A and β_B as well, to form activin C, activin AC and activin BC (not shown in the diagram). The inhibin α subunit encoded by the INHA gene can form inhibin A and inhibin B with the β_A or β_B subunit, respectively. Inhibin can be an activin inhibitor by competing for the activin receptor. Figure modified from Hedger et al. (2011).

stimulating or inhibiting the release of FSH (Eto et al. 1987). Possessing activity similar to that of inhibin, follistatin initially was identified as an inhibitor of FSH, but follistatin

has a different mechanism of action to inhibin, inhibiting FSH through binding and neutralising activin's stimulatory activity, whereas inhibin blocks activin at the receptor level (Nakamura et al. 1990; Ueno et al. 1987).

Activins arise from five distinct subunits and genes, termed β_A-β_E. Activin A is the most well-studied activin dimer, consisting of a homodimer of two activin β_A subunits (Greenwald et al. 2004; Harrington et al. 2006; Lin et al. 2006)(Fig. 1). Activin A is highly conserved with 100% conservation between the human, monkey, rat and mouse protein (Phillips 2003).

The activin A dimer is produced as a precursor with a cleavable pro-region (Walton et al. 2012)(Fig. 2). The activin A precursor comprises 402-426 amino acids and is processed to the mature bioactive protein (116 amino acids) by cleavage of the C-terminal region by acid hydrolysis (Gray and Mason 1990; Hedger et al. 2011; Huylebroeck et al. 1990). Similar to TGF-β, the pro-region of activin A is important for folding, formation of disulphide bonds and secretion of the active activin A protein (Gray and Mason 1990). The major circulating form of activin A is the processed mature dimer (Fig. 2), which is different to that seen for TGF-β, where the pro-region remains tightly attached (Sha et al. 1991). The pro-domain helps stabilize the activin dimeric structure before cleavage. The extracellular pro-domain allows activin A bind to heparan sulphate proteoglycans (HSPGs) (Walton et al. 2012).

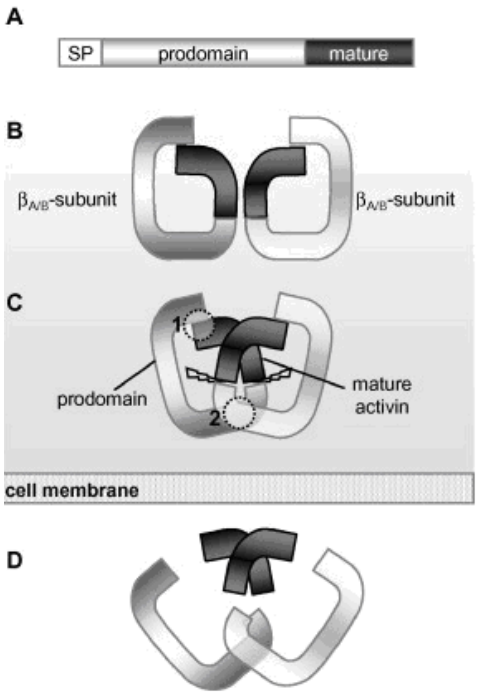

Figure 2 Synthesis and release of mature activin A and B. The precursor of activin A/B consists of prodomain and mature regions (A). The prodomain is cleaved from the precursor after formation of the dimeric activin protein (B and C) and then mature activin protein is secreted from the cell (D). SP- signal peptide. The pro-domain helps stabilize activin dimeric structures before cleavage. The extracellular pro-domain helps activin A bind to HSPGs. Figure reproduced from Walton et al. (2012).

Other activin/inhibin subunits

Apart from the activin β_A subunit, the activin/inhibin family includes the activin $\beta_{B\text{-}E}$ subunits and the inhibin α subunit. All the activin β subunits have around 50% homology with each other (Sekiyama et al. 2009). All the subunits can form dimers with other subunits to form heterodimers, while the β subunits can also form homodimers (Fig. 1). For example, the expression of activin AB (β_A and β_B subunits), AC ($\beta_A\beta_C$), AE ($\beta_A\beta_E$), CE ($\beta_C\beta_E$) (Muenster et al. 2005), as well as BC ($\beta_B\beta_C$) have been detected (Evans et al. 1997). Activin A and B have some common functions, but there is evidence that they also

have divergent functions due to their different affinities and ability to interact with different receptor subtypes (Corrigan et al. 1991). Activin C and possibly activin E act as competitive antagonists via inhibiting activin A or B formation or activin receptor binding (Gold and Risbridger 2012).

Activin B consists of two activin β_B-subunits, with approximately 64% homology with activin β_A at the amino acid level (Mason et al. 1985; Mason et al. 1986). Activin B is less studied than activin A partly because of the previous lack of a sensitive and specific assay and activin B also appears to be less bioactive and less widely expressed. Recently, a two-site enzyme-linked immunosorbent assay (ELISA) for activin B protein has been developed (Ludlow et al. 2009). In contrast, an ELISA for activin A has been available and utilised for a number of years (Knight et al. 1996). Both activin A and B assays measure both free and follistatin-bound activin A or activin B dimers and have no significant cross-reaction with other isoforms of activin (Knight et al. 1996; Ludlow et al. 2009). Follistatin measurement in our laboratory utilizes a radioimmunoassay (RIA), with human recombinant follistatin 288 as standard and tracer. Interference of activins is minimized by the use of dissociating reagents (O'Connor et al. 1999). This assay therefore measures total follistatin (Ludlow et al. 2009).

The activin β_C subunit gene has antagonistic activity, since both activin homo- and hetero-dimers containing these subunits bind to activin type II receptors but lack receptor signalling activity (Muenster et al. 2005). Activin β_D has only been found in the *Xenopus* genome (Oda et al. 1995; Phillips 2003). Activin E plays roles in cell growth and apoptosis in some cell types, but activin E knockout mice have not shown any abnormalities (Lau et al. 2000; Rodgarkia-Dara et al. 2006). Nevertheless, mice

genetically overexpressing activin E were found to have notable aggressive behaviour (Lau et al. 2000; Rodgarkia-Dara et al. 2006; Sekiyama et al. 2009).

The inhibin α subunit has less similar homology to the activin subunits, but is able to form bioactive dimers with either activin β_A or β_B subunits to form heterodimers, inhibin A and inhibin B (Phillips 2003) (Fig. 2). The inhibin α subunit is most highly expressed in the gonads, with lower levels expressed in extra-gonadal tissues, such as bone marrow, kidney, pituitary, placenta, adrenal gland and the central nervous system (CNS) (Meunier et al. 1988). Inhibin α subunit gene knockout animals developed gonadal tumour and cachexia and adrenal glands tumours (Matzuk et al. 1994). These tumours were associated with increased activin A and B protein secretion (Coerver et al. 1996). This evidence implicates activin A and B as having actions to stimulate the development of tumours.

The activin A gene and gene regulation

The activin β_A gene is located on chromosome 7 in the human and on chromosome 13 in the mouse (Phillips 2003). The human activin β_A gene has three exons separated by two introns (Tanimoto et al. 1996; Yoshida et al. 1998) (Fig. 3). It has been shown that the activin β_A gene transcription start site is located 212 nucleotides upstream of the translation start codon (Dolter et al. 1998). There are two sites in the enhancer region that are important for full promoter activity, including several activator protein-1 (AP-1; c-Jun homodimer or c-Jun/c-Fos heterodimer) binding sites and a conserved cyclic AMP-responsive element (CRE)-like site (C site, −123 to −116), which can interact with the nuclear factors, CREB/ATF (Ardekani et al. 1998; Tanimoto et al. 1996) (Fig. 3). The proximal promoter contains a binding site for c-MAF, which is a type 2 helper T cell

(Th2)-specific transcriptional factor that can work with NF-AT to stimulate β_A gene transcription (Ogawa et al. 2006). This was confirmed by a study that showed that the activin β_A gene was elevated in Th2 cells but not type 1 helper T (Th1) cells (Ogawa et al. 2006). As in α subunit gene and β_B gene, several cAMP- responsive transcription factor AP-2 sites have been identified in the β_A gene 5' promoter regions (Thompson et al. 1994). Both phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA) 1, and cAMP analogues, such as 8-bromo-cyclic AMP (8-Br-cAMP), stimulate human activin β_A subunit gene expression (Schneider et al. 2000).

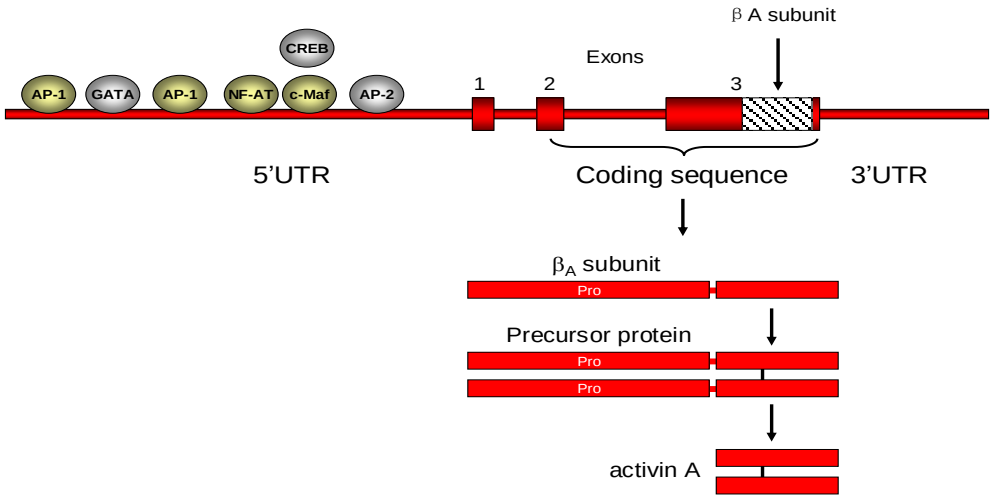

Figure 3 Activin β_A subunit gene and promter organization and synthesis of activin A. The activin β_A subunit gene has 3 exons, the enhancers have pro-inflammatory transcription factors activator protein-1 (AP-1) and AP-2 binding sites, nuclear factor of activated T-cells (NF-AT) and c-Maf, as well as binding sites for cAMP-responsive element binding protein (CREB) and GATA. The activin A precursor and mature coding sequence is encoded by exons 2 and 3 and the mature activin A dimer is the products of a disulphide-linked homodimer of the precursor after protein cleavage. Figure modified from Hedger and Winnall (2012).

Biological activities of activin A

As a member of the TGF-β superfamily, activin A has a broad range of bioactivities. Activin A regulates immunity, inflammation and fibrosis, as well as proliferation, differentiation and apoptosis in many cell types (Phillips 2003).

The activin receptors

Similar to other TGF-β superfamily members, activin receptors consist of two types of serine-threonine trans-membrane kinase receptors, termed type I and type II receptors (Piek et al. 1999). The activin receptors consist of an extracellular ligand-binding domain, a short transmembrane domain and a large cytoplasmic serine/threonine kinase domain, which is responsible for activating intracellular signalling (Sako et al. 2010). Each dimer of activin binds the two receptor subunits of the mature receptor complex (Phillips 2003).

There are two type I activin receptors, ActRIB or activin receptor like kinase-4 (ALK -4) and ActRIC (ALK-7). The so-called ActRIA receptor (ALK-2) is not a functional receptor for activin signalling (Attisano et al., 1993). ActRIB is the type I receptor for activin A and activin B (Attisano et al. 1993), but the ALK-7 receptor binds activin B and activin AB, but not activin A (Tsuchida et al. 2008) (Fig. 4). There are two forms of type II receptors, ActRIIA and ActRIIB, which have differing activin ligand binding affinities (Attisano et al. 1992; Xu et al. 1994). The type II receptors are able to bind to activin directly. After binding with an activin dimer, the type II receptors recruit and phosphorylate type I receptors on serine and threonine residues located in a conserved juxtamembrane 'GS' domain to form the active signalling complex (Attisano et al. 2001) (Fig. 4). The type II receptors cannot activate intracellular signalling without binding to a type I receptor (Donaldson et al. 1999). ActRII-null mice have reproductive defects such as a lack of corpora lutea in the ovaries of female mutant mice and decreased tubule size from testes of male mutant mice, but no cleft palate, which is a phenotype of the activin β_A gene knockout and of β_A/β_B double knockout mice (Matzuk et al. 1995a). An interpretation of these results is the suggestion that activin receptors are able to bind with other ligands of the TGF-β family (Phillips 2003).

Regulation of activin signalling at the receptor level also involves several accessory molecules. Inhibin, containing one β subunit, antagonises activin signalling by binding to the type II receptor, but failing to activate downstream signalling - thereby inhibin acts as a functional inhibitor of activin binding and signalling (Xu et al. 1995). Another antagonist is the activin membrane bound inhibitor, BAMBI (bone morphogenetic protein and activin membrane-bound inhibitor), which can bind with the type II receptors, but act as a type I pseudoreceptor, because it lacks the intracellular kinase domain of the type I receptors, resulting in a failure to activate signalling (Onichtchouk et al. 1999). Other proteins that affect activin signalling through the interactions between activin and/or its receptor subunits include endoglin/CD105 and the GPI-linked membrane protein, Cripto (Barbara et al. 1999; Gray et al. 2003). The interaction between activin and the binding protein, follistatin, will be discussed later in this review.

Activin A intracellular signalling

The Smad proteins act as intermediates between the TGF-β superfamily receptors and the induction of gene expression. There are three kinds of Smad molecules involved in activin signalling: receptor-regulated Smads (R-Smad 2 and 3), inhibitory Smads (I-Smads 6 and 7) and the common partner Smad (Co-Smad) for all the receptor activated Smads, Smad 4 (McDowall et al. 2008). After binding with activin, phosphorylated type I receptors recruit and phosphorylate the R-Smad proteins, Smads 2 and 3, by the serine/threonine kinase domain of the ligand-activated receptor complex for phosphorylation of serine and threonine residues of R-Smad ((Itoh et al. 2002) (Fig. 4)). A FYVE domain protein, the Smad anchor for receptor activation (SARA), recruits R-Smads to the activated activin receptor complex (Tsukazaki et al. 1998). After

phosphorylation, the R-Smads disconnect from SARA (Itoh et al. 2002), and then bind to Smad4 (Souchelnytskyi et al. 1997) to initiate downstream signalling. The Smad complexes move to the nucleus where they bind with DNA binding partners for nuclear translocation and upregulation of downstream target genes (Tsukazaki et al. 1998) (Fig 4). Smad3 and/or Smad4 are able to recognize and bind specific DNA sequences of a Smad binding element (SBE) (Dennler et al. 1998; Yingling et al. 1997; Zawel et al. 1998). However, Smad complex binding with DNA requires DNA binding partners to improve the affinity and specificity. A winged-helix fork-head activin signal transducer (FAST)-1, a transcription factor that can bind with specific elements in the promoters of target genes, has been identified as a Smad DNA binding partner, which can interact with the Smad 2/Smad 4 complex (Chen et al. 1997).

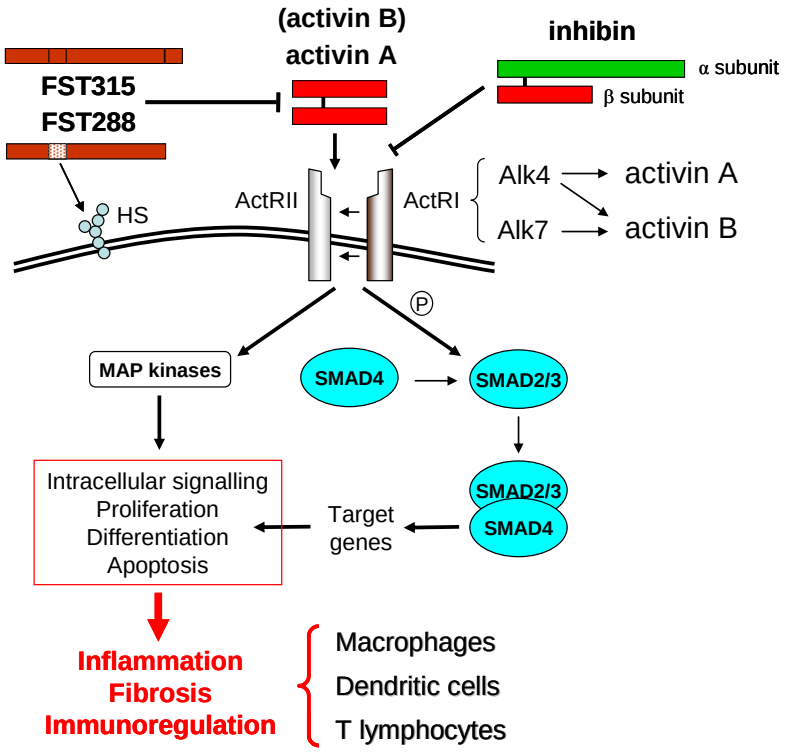

Figure 4 Activin A intracellular signalling. After binding with the activin A or activin B dimer, an ActRII receptor subunit phosphorylates the corresponding ActRI. Activated ActRI recruits and phosphorylates Smad 2 and/or Smad3, which in turn forms a complex with Smad4. This Smad complex translocates to the nucleus to regulate gene transcription. Follistatin inhibits activin signalling by binding with activin dimers and preventing the activation of intercellular signalling. Inhibin interferes with activin signalling by competitively binding the activin receptor. Activin A also stimulates MAP kinase signalling, activating proliferation and apoptosis and activation of MAP kinase-responsive transcription factors. Figure modified from Hedger et al. (2011).

Smad6 and Smad7 are inhibitory Smads, which have the opposite effects to the R-Smads, and inhibit activin signalling by blocking the phosphorylation of Smad2 protein by the type I receptor kinase and inhibiting the formation of Smad2/Smad4 complexes (Hayashi

et al. 1997; Imamura et al. 1997). Smad 6 and Smad 7 also have differential inhibitory effects on BMP-2- and activin A-mediated signalling in B lineage cell lines (Ishisaki et al. 1999). The three forms of TGF-β (TGF-β1-3) also signal via Smad2, Smad3 and Smad4, but through their own receptor subtypes. The BMP/GDF subfamily utilises Smads 1, 5 and 8 as their R-Smads (Wang et al. 2009).

Regulation of activin A in vivo

It has been established in various studies that activin A is increased in the circulation and in various tissues during acute or chronic inflammatory disorders, including infections due to viruses, bacteria and other micro-organisms (Jones et al. 2004a; Patella et al. 2001), non-infectious inflammatory bowel disease (Hübner et al. 1997), surgery (Chen et al. 2011a), ischaemic brain disease (Wu et al. 1999) and trauma (Phillips et al. 2006). Furthermore, activin A was found to increase in the cerebrospinal fluid (CSF) during acute meningitis (Ebert et al. 2006), in the lung and serum during acute asthma (Karagiannidis et al. 2006) and in skin wounds after burns (McLean et al. 2008). Inflammatory cytokines, such as IL-1, IL-8, IL-10, interferon- γ (IFN-γ) and TGF-β are able to stimulate expression of activin A mRNA in synoviocytes (Dolter et al. 1998). IL-1β and TNF-α were found to stimulate activin β_A subunit mRNA and protein secretion in endometrioma stromal cells (Yoshino et al. 2011) and TNF-α induced the release of activin A from human neutrophils in recent studies from our own laboratory (Chen et al. 2011b).

Several studies have confirmed that the injection of LPS is able to stimulate the release of activin A protein into the circulation (Jones et al. 2000; Jones et al. 2004a), and that the increase of activin A release is dependent upon TLR4-induced signalling (Jones et al.

2004a; Jones et al. 2007). After binding LPS, TLR4 recruits an adaptor protein called MyD88, and then activates the transcription factors, NF-κB and AP-1, which initiate transcription of inflammatory genes (Jenkins and Mansell 2010; O'Neill and Bowie 2007), as well as new activin β_A subunit mRNA transcription and protein synthesis (Arai et al. 2011; Norwitz et al. 2002). The key inflammatory cytokine, IL-1 also acts by recruiting MyD88 to induce activin downstream signalling (Jenkins and Mansell 2010; O'Neill and Bowie 2007). Apart from the MyD88-dependent pathway, activation of TLR3 and 4 can also trigger inflammatory cytokine production and release by acting through other adaptor proteins, TRIF and MAL (O'Neill et al. 2003; Reis et al. 2011). Activation of these adaptors triggers the induction of transcription factors, such as IRF3, that stimulate type 1 IFN (α and β) production (Hertzog et al. 2003; O'Neill and Bowie 2007).

In addition to these inflammatory stimuli, activation of PKC by phorbol esters also stimulates activin β_A subunit mRNA expression and secretion, which has been confirmed by the reduced production of activin A in human bone marrow stromal cells treated with a PKC inhibitor (Scicchitano et al. 2008). Activation of the cAMP-dependent PKA pathway has also been found to increase production of activin A protein (Miyanaga et al. 1993; Tuuri et al. 1996).

TNF-α is also able to up-regulate the expression and secretion of activin A in bone marrow stromal cells and human blood neutrophils (Chen et al. 2011b; Shao et al. 1992). Other cytokines able to stimulate the expression and secretion of activin A are granulocyte-monocyte colony stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PFGF) (Chen et al. 2011b; Pawlowski et al. 1997; Shao et al. 1992; Uchimaru et al. 1995; Wang

and Ge 2004). In addition, vasoactive agonists such as angiotensin II and α-thrombin are able to stimulate activin β_A subunit mRNA in rat aortic smooth muscle cells (Pawlowski et al. 1997). In contrast with the pro-inflammatory cytokines that up-regulate β_A subunit mRNA or activin A protein expression, anti-inflammatory steroids, such as glucocorticoids, inhibit the expression and release of activin A in several cell types (Shao et al. 1998; Yu et al. 1996).

Sources of activin A

Although activin A was first isolated from the gonads as an FSH regulatory protein (Eto et al. 1987), it is now known that the gonads are not the only or even the major source of circulating activin A (Sakai et al. 1992). Circulating activin A levels are not affected by gonadectomy (McFarlane et al. 1996), and both intact and gonadectomised animals have elevated release of activin A in response to LPS injection (McClure et al. 2005). Because of its widespread functions, even in normal conditions, activin A is estimated to be synthesised or stored by almost every cell type in the body (Phillips 2003), and therefore most tissues have the ability to be the candidate source of activin A in the circulation. Some particular cell types that are major producers of activin A, including myeloid cells, epithelial cells, endothelial cells and fibroblasts, which are components of most tissues (Delneste et al. 2007; Parker et al. 2005; Phillips et al. 2009). Further, the source of activin β_A subunit mRNA has been documented (Dohrmann et al. 1993) and several studies have shown that the activin β_A subunit gene is widely expressed under normal conditions (Meunier et al. 1988; Tanimoto et al. 1992; Tuuri et al. 1994), Nonetheless, there has been no comprehensive quantitative examination of this distribution of activin subunit mRNAs or mature protein, or the response to LPS administration *in vivo* and this is lacking in the literature.

Follistatin and the regulation of follistatin

Follistatin is a monomeric glycosylated single-chain protein, which was first identified as a gonadal product isolated from bovine or porcine follicular fluid with inhibin-like bioactivity, but different in molecular structure from inhibin (Esch et al. 1987; Robertson et al. 1987). As follistatin is a monomeric protein and the activins are dimeric proteins, it has emerged that two follistatin molecules can bind with one activin dimer (Ogawa et al. 2000; Phillips et al. 2009) (Fig. 5). Follistatin itself consists of three homologous 10-cysteine domains (follistatin domains 1, 2 and 3) and a unique N-terminal (Inouye et al. 1991; Sidis et al. 2001). Follistatin domains 1 and 2 are important for activin binding (Harrington et al. 2006) (Fig. 5). The follistatin gene consists of five introns and six exons, with two mRNA transcripts produced by alternative splicing, giving two proteins: FS288 with 288 amino acids and FS315 with 315 amino acids (Michel et al. 1990; Shimasaki et al. 1988). FS315 is believed to be the main circulating form, and FS288 is more likely to be associated with cellular surfaces (Schneyer et al. 2004). The binding to cell surfaces by FS288 is due to the ability to bind to cell surface HSPGs, while in FS315 the binding site is masked by the C-terminal extension (Sugino et al. 1993). After binding to activin, a C-terminal conformational change enables FS315 to bind to heparin/heparan sulphates due to the uncovering of the binding site (Lerch et al. 2007; Sugino et al. 1993) (Fig. 5). When heparin, an anticoagulant, is injected at therapeutic doses into sheep or humans, activin and follistatin are released from the cell surface of tissues and rapidly increase in the circulation (Klein et al. 1996b; Phillips et al. 2000). Activin and follistatin that is bound by cell surfaces are removed by a lysomal degradation pathway, which suggests that cells may expose FS288 on their surface as a mechanism to control activin A activity locally (Hashimoto et al. 1997).

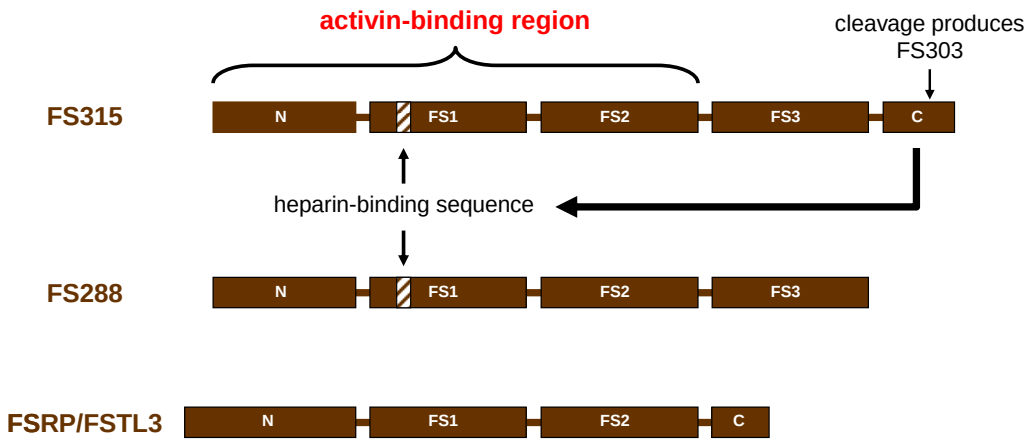

Figure 5 Follistatin/FSRP protein organization. The follistatin molecule consists of three FS domains and an amino-terminal (N-terminal). FS315 has a C-terminal tail extension that normally obstructs the heparin-binding motif in the FS1 domain. Activin binds with follistatin at the N-terminal region and the first two FS domains. The heparin-binding motif enables follistatin to bind to both heparin and HSPGs on cell surfaces. After FS315 binding with activin, a C-terminal conformational change uncovers the heparin-binding site. FSRP has two follistatin domains and can bind with activin, but at lower affinity, and does not bind to HSPGs. FS303 is a cleavage product of FS315 that has been found in biological samples, but its functional significance is unknown at present. FS: follistatin. Figure modified from Hedger et al. (2011).

There are some other proteins that have follistatin domains (Schneyer et al. 2003). Another protein homologous to follistatin, FSRP, can also antagonise activin signalling, as it also contains follistatin domains in its structure, but its affinity for the activins is 20-fold lower than the affinity of follistatin for the activins (Harrington et al. 2006; Tsuchida et al. 2000) (Fig. 5), Although both inhibin and follistatin can antagonize activin

signalling, the mechanism of interaction by which this is achieved is different. This was confirmed by the evidence that an inhibin antiserum could not neutralise follistatin's effect on the content of FSH in pituitary cell cultures (Esch et al. 1987; Robertson et al. 1990). Follistatin has a high affinity to bind with activins, which is similar to that of activins binding with activin receptors and is nearly irreversible, so that the activins cannot bind with their specific receptors to initiate signalling (Phillips & de Kretser, 1998). Compared with activin A, the affinity of activin B for follistatin is 10-fold lower (Schneyer et al. 2003). Because inhibin has only one activin β subunit, it is able to bind with follistatin as well, but with a much lower affinity (Shimonaka et al. 1991). Further, the biphasic release of activin into the circulation after a challenge with LPS occurs much earlier than follistatin release, indicating that the release of activin A is not from activin-follistatin complexes from the cell surface, but is due to free activin A protein (Jones et al. 2004b; Jones et al. 2007).

The neonatal lethality of follistatin-null mice reveals the biological importance of follistatin (Matzuk et al. 1995b). These animals usually die within 24 hours of birth, because of insufficient intercostal and diaphragm muscles leading to an inability to breathe normally; pups become cyanotic. However, follistatin overexpression transgenic mice have a different phenotype from that of the activin β_A subunit knockout, in that the former can lead to induction of neural tissue or muscle hypertrophy and hyperplasia (Li et al. 2011; Matzuk et al. 1995b), whereas the latter showing a cleft palate defect (Matzuk et al. 1995a).

Regulation of follistatin

In vitro, it has been shown that inflammatory factors, such as activin A, activin B and IL-

1β, can stimulate the release of follistatin from pituitary cells cultures, whereas inhibin A decreases follistatin mRNA level and follistatin can inhibit its own mRNA accumulation in pituitary cells (Bilezikjian et al. 1996; Bilezikjian et al. 1998; Dalkin et al. 1996). There are other data that show follistatin is possibly regulated by other cytokines, such as TNF-α and IFN-γ (Abe et al. 2001; Keelan et al. 2000; Michel et al. 1996; Phillips et al. 1996). Meanwhile, in conditions with activin A elevation, such as surgical trauma or septicaemia, follistatin has also been found to be elevated (Jones et al. 2004b; Klein et al. 1996b). These lines of evidence suggest that follistatin is not only regulated by activin A, but also by other inflammatory cytokines.

In addition to the above, testosterone inhibits, while gonadotropin-releasing hormone stimulates, follistatin production or expression in pituitary cells (Besecke et al. 1996; Bilezikjian et al. 1996). It is also reported that follistatin is increased in women with polycystic ovary syndrome (PCOS) and follistatin is positively correlated with obesity, insulin resistance and the inflammatory marker, CRP, in these women (Chen et al. 2009).

I.3 Inflammation, Inflammatory Responses and Immunity

Inflammatory response and immunity

In general, inflammatory responses are the body's reaction to harmful stimuli. Inflammation is the first immune response to infection or stimuli. The inflammation can be acute or chronic inflammation, and the triggers may be due to infectious agents or to non-infectious (or sterile) causes (Eissa et al. 2010). The latter include the noninfectious inflammatory response initiated by molecules released by tissue damage, called danger-associated molecular patterns (DAMPs), and an infectious pathogen inflammatory response induced by pathogen-specific molecules, called pathogen-associated molecular patterns (PAMPs) (Janeway 1989). The infectious causes include bacteria, viruses and

fungi, whereas the non-infectious causes involve physical stress or damage, as observed in surgery, haemorrhage, trauma, pulmonary embolism, burns, drug overdose, diabetic ketoacidosis and anaphylaxis (Eissa et al. 2010). The inflammatory response consists of local and systemic responses. In general, it involves an increase in blood flow and capillary permeability, invasion of inflammatory cells, such as neutrophils and mononuclear cells, activation of coagulation and the process of tissue damage, and the protection process of tissue repair and fibrosis during healing. Further, pro-inflammatory cytokines and other inflammatory mediators are increased, such as TNF-α, IL-1, IL-6, CRP, complement, prostaglandins, several enzymes, reactive oxygen and nitrogen, (Eissa et al. 2010; Levi and van der Poll 2010), which may impact upon activin or be regulated by activin. In addition, the inflammatory response involves not only immune cells, but also fibroblasts and endothelial cells in the inflamed tissues (Levi and van der Poll 2010).

Immunity can be thought of as adaptive immunity and innate immunity. Adaptive immunity is the immunity for specificity and memory (Akira et al. 2001). In this review, the emphasis is on innate immunity, which is the rapid, less-specific immune response initiated when macrophages and other leukocytes, such as neutrophils, recognise the pathogen or other foreign substance (Akira et al. 2001). The body undertakes a defensive response by increased blood flow and movement of macrophages and leukocytes to the local area to recognize, bind, engulf and digest the pathogen (Aderem and Ulevitch 2000; Akira et al. 2001; Janeway and Medzhitov 1998).

There is considerable evidence that activin A has important effects on both pro- and anti-inflammatory responses during inflammation (Hedger et al. 2011; Phillips et al. 2009). For example, activin can activate monocyte/macrophages functions by stimulating the

development of inflammatory mediators, such as IL1β, TNFα, IL6, nitric oxide and prostaglandin E2, and also down-regulate the inflammatory functions of monocyte/macrophages by inhibiting production of IL6, an important T and B cell growth factor (Hedger et al. 2011).

LPS and LPS-induced inflammation, tissue response and intracellular signalling

LPS consists of an O-specific chain, a core oligosaccharide, and a lipid moiety, known as lipid A, which is the key component determining the endotoxicity of the molecule (Raetz et al. 1991; Rietschel et al. 1994). During a bacterial infection, LPS binds to LBP, a lipid transfer protein, to form a LPS-LBP complex, which transfer LPS to a binding site on soluble Cluster of Differentiation 14 (sCD14) (Hailman et al. 1994; Yu and Wright 1996). LPS is unable to bind directly to its receptor, TLR4, and interaction of LPS and TLR4 requires the additional involvement of an accessory protein, called MD2, to form a receptor/signalling complex of CD14-MD2-LPS-TLR4, which is able to activate intracellular signalling pathways. This signalling occurs through intracellular proteins, such as the adaptor proteins MyD88 and TIRAP (Scott and Billiar 2008) (Fig. 6). Following LPS binding to TLR4, MyD88, an adaptor protein in the Toll/IL-1 receptor family signalling pathway, is recruited and binds to the intracellular domain of TLR4. This complex recruits and phosphorates IRAK and IRAK2 (O'Neill et al. 2003). The phosphorylated IRAKs dissociate from MyD88 and form another complex with TRAF-6, which results in activation of NF-κB. Activated NF-κB moves into the nucleus to regulate gene expression. The degradation of IκB and the activation of proteasome as well as the TRAF6-IRAK complex also are able to phosphorylate and activate TAK-1, thereby activating the JNK/stress-activated protein kinase or p38 (McDermott and O'Neill 2002; O'Neill et al. 2003; Reis et al. 2011). This causes a rapid induction and release of

inflammatory cytokines, chemokines and biologically active lipids from several cell types, especially monocyte/macrophages and endothelial cells (Caroff et al. 2002; Rietschel et al. 1996; Yu and Wright 1996). In addition to MyD88, LPS also can bind with another adaptor protein, TIRAP (O'Neill et al. 2003) (Fig. 6). Activin A and follistatin are also released in an LPS-induced inflammatory response (Jones et al. 2004a). Most inflammatory genes are regulated primarily at the transcriptional level, such as IL-1β, IL-6 and TNFα (Falvo et al. 2010; Perez et al. 1999; Rambaldi et al. 1993). The expression of both IL-1β and TNFα are rapidly induced, and the protein appears within one hour after LPS-treatment. Activin A also appears very rapidly, but its regulation in this context is not well-understood. In fact, many studies suggest that transcriptional expression of activin A in cells, such as the macrophages, endothelial cells or Sertoli cells, takes many hours (Erämaa et al. 1992; Hedger et al. 2011; Kazutaka et al. 2011; Wang et al. 2008; Wilson et al. 2006); how can this rapid release be explained? Could activin be stored, like histamine is stored and released by neutrophils and mast cells very rapidly (Nagai and Koda 1979)? This lack of knowledge will be examined in this thesis, where the regulation of activin A release will be examined, and whether it is from new synthesis of protein or pre-stored protein, or both.

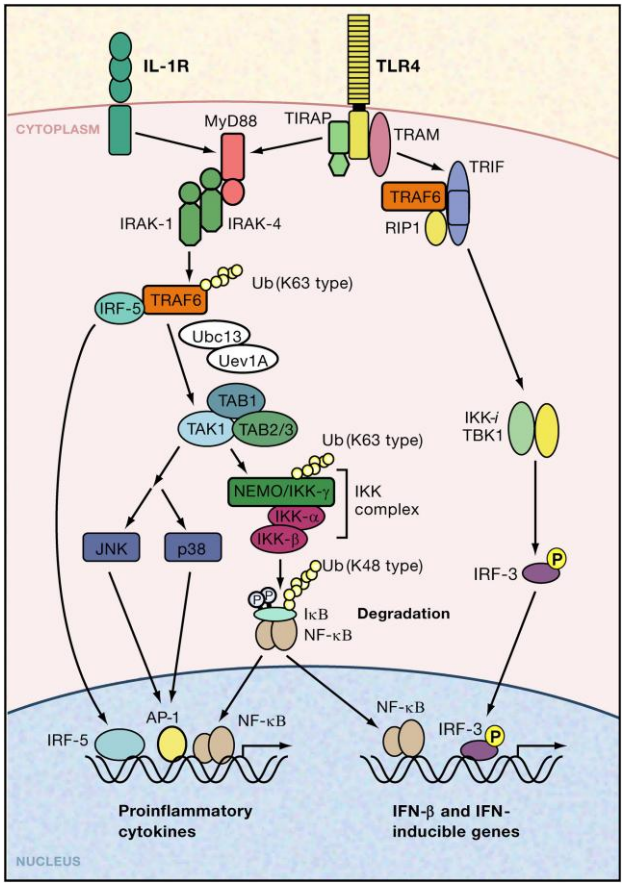

Figure 6 LPS signalling is initiated by binding to TLR4. The TLRs share common signalling pathways with IL-1 and the IL-1 receptor (IL-1R). Recruitment of TIR-domain-containing adaptors, MyD88 and TIRAP, leads to formation of a complex of IRAKs, TRAF6, and IRF-5. TRAF6 acts as an E3 ubiquitin ligase and ubiquitination of TRAF6 and nuclear factor-κB essential modulator (NEMO) activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IκB undergoes ubiquitination and degradation by the proteasome. Freed NF-κB translocates into the nucleus and initiates the expression of pro-inflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1. TLR4 also triggers the MyD88-independent, TRIF-dependent signalling pathway via TRIF-related adaptor molecule (TRAM) to induce type I IFNs via NF-κB and IRF-3. Figure reproduced from Akira et al. (2006).

Release of Activin A in acute inflammatory responses

Circulating levels of activin A increase rapidly after an LPS-induced challenge through activation of TLR4 (Muzio et al. 1998). Animal models (such as the sheep and mouse) demonstrate that systemic injection of LPS results in the rapid appearance in the bloodstream of inflammation-responsive proteins known as cytokines and an increase in core body temperature (Jones et al. 2004a). After injection of LPS into the jugular vein of sheep, activin A release into the circulation was a very early event in the inflammatory cascade, preceding the release of TNF-α and following soon thereafter the increase in core body temperature (Jones et al. 2004a).

Activin and clinical inflammatory syndromes

As well as responses in animal models of acute inflammation using LPS treatment, the clinical relevance of activin A in inflammatory syndromes has been confirmed in infections such as meningitis (Wilms et al. 2010) and in septicaemia (Phillips et al. 2001). Moreover, activin A is also elevated in many other acute or chronic inflammatory syndromes, including asthma (Kariyawasam et al. 2009), pulmonary hypertension (Yndestad et al. 2009), angina (Smith et al. 2004), heart failure (Yndestad et al. 2004), inflammatory bowel disease (Hübner et al. 1997), gastric ulcers (Becker et al. 2003), viral hepatitis (Patella et al. 2001), liver failure (Hughes and Evans 2003), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (El-Gendi et al. 2010), burns injuries (McLean et al. 2008) and pre-eclampsia (Yu et al. 2011). All of these disorders have the common characteristics of elevated pro-inflammatory cytokine levels, such as CRP and/or TNF-α and an enhanced ESR. Furthermore, blockade of activin A by follistatin can affect pro-inflammatory cytokine release in LPS-induced acute inflammation in mice (Jones et

al. 2007), which suggests that activin A is a core component of any inflammatory response and not just specific to a particular syndrome. In clinical septicaemia, patients with higher systemic activin A concentrations were found to more likely suffer from complications and even have higher mortality, while animal studies show that injection of follistatin, to block the effects of activin A, can improve survival following LPS administration to induce septic-like shock (Jones et al. 2007; Michel et al. 2003).

Activin A, inflammation and immunity

Activin A has been found to have several important roles in the immune system. For example, activin A is able to inhibit both T and B lymphocytes and enhance the differentiation from monocyte lineages towards a macrophage phenotype (Pierce et al. 1992; Semitekolou et al. 2009; Zipori and Barda-Saad 2001). Furthermore, during acute inflammation, activin A stimulates bone marrow-derived macrophage and monocytes to increase the production of inflammatory factors, such as TNF-α, IL-1β and IL-6 (Hedger et al. 2011). Activin A also enhances the movement of macrophages towards the basement of the membrane to stimulate the inflammatory reaction (Ogawa et al. 2000). On the other hand, activin A also has anti-inflammatory functions during immune reactions. Activin A blocks IL-6-stimulated acute phase protein production in the HepG2 cell line(Russell et al. 1999). T helper (Th) cells are the T lymphocyte which coordinate maturation or differentiation of other white blood cells, or activate with different Th type cells secreting different cytokines to modulate different immune processes (Gutcher and Becher 2007). The activin βA subunit gene has been found in Th2 cells but not in Th1 cells (Ogawa et al. 2006). Activin A helps macrophages switch from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, thereby reducing inflammation (Ogawa et al. 2006).

Bone marrow haematopoiesis

Bone marrow is the source of neutrophils and is also the source of all the blood cells, including all immune cells. The process of blood cell formation from bone marrow has been called haematopoiesis, which includes pluripotent haematopoietic stem cells deriving all the blood cells progenitors and all the blood lineages derived from progenitor cells (Blank et al. 2008; Metcalf 2008) (Fig. 7). The progenitors include common lymphoid progenitor cells which develop B and T lymphocytes and NK cells, common myeloid progenitors which develop polymorphonuclear leukocytes, megakaryocytes which develop platelets, and erythroblasts which develop erythrocytes. Mature blood cells are released from the bone marrow to the blood and then are transferred to either lymph nodes or tissues via the blood system or lymphatic system (Blank et al. 2008; Metcalf 2008) (Fig. 7). The pluripotent haematopoietic stem cells and progenitor cells are supported by surrounding bone stromal cells and bone marrow stromal cells, including fibroblastic cells, endothelial cells, and macrophages (Ding et al. 2012; Taichman 2005; Wilson and Trumpp 2006).

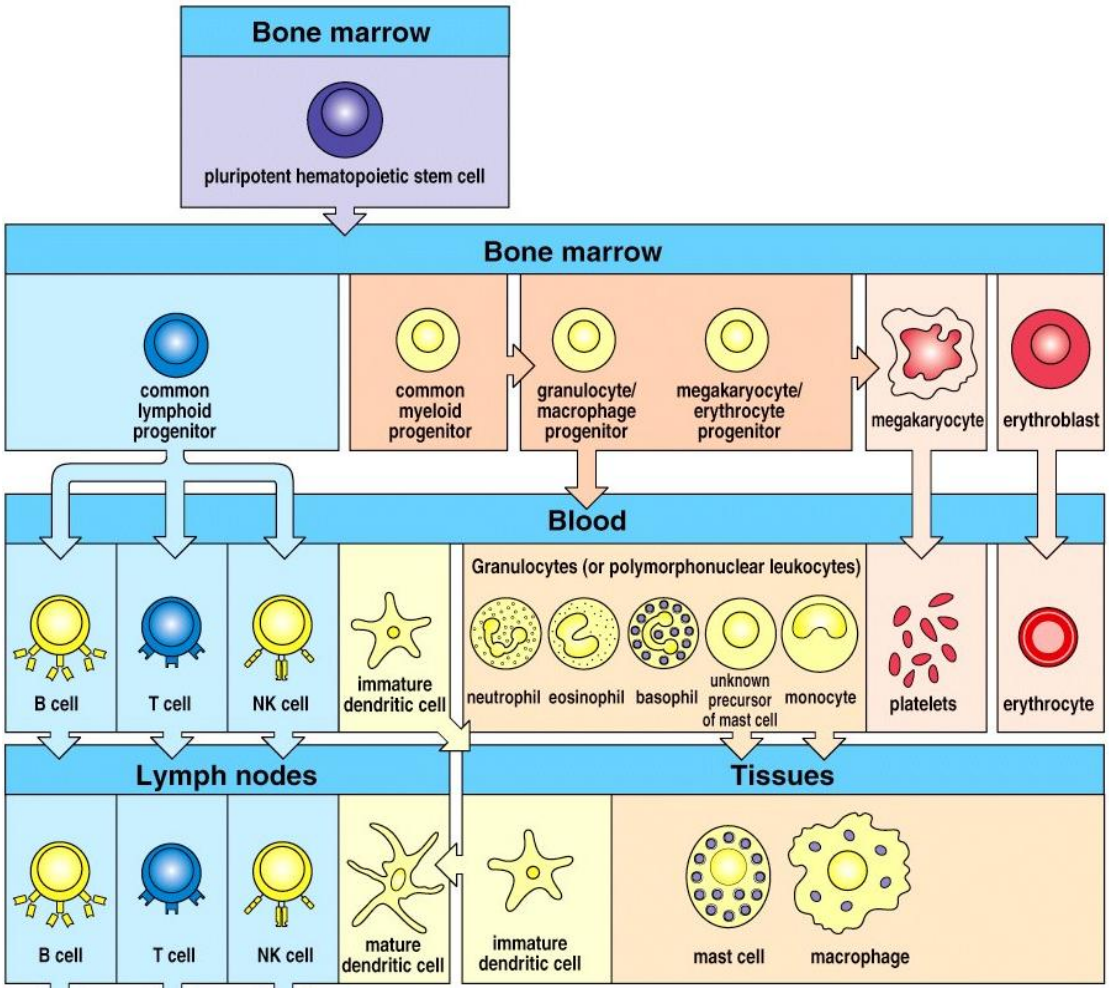

Figure 7 Bone marrow haematopoiesis. Bone marrow haematopoiesis includes pluripotent haematopoietic stem cells deriving all the blood cells progenitors and all the blood lineages derived from progenitor cells. The progenitors include common lymphoid progenitor cells which develop B and T lymphocyte and NK cells, common myeloid progenitors which develop polymorphonuclear leukocytes, megakaryocytes which develop platelets, and erythroblasts which develop erythrocytes. Thereafter, mature blood cells released from the bone marrow enter the bloodstream and reach either lymph nodes or tissues via the circulation. Figure reproduced from Immunobiology, 6th Edition Janeway et al. (2005)

Neutrophils and inflammation

The neutrophil is one of the most important immune cells in the innate immune response (Normark et al. 2001). In acute inflammation, neutrophils can accumulate in tissues and in the circulation. It has been shown that neutrophils express the TLRs (Parker et al. 2005; Phillips et al. 2009). It has been noted that activin A immunostaining is detected in neutrophils (Leung et al. 1998). Recent data from our laboratory have shown that human blood neutrophils contain 20-fold higher levels of pre-stored activin A than blood mononuclear cells (Chen et al. 2011b). The same study showed that TNF-α, but not LPS itself, could directly trigger the release of activin A from human blood neutrophils *in vitro* (Chen et al. 2011b). These finding raise the question whether activin A stored within neutrophils could be a source of activin A during acute inflammation.

It has been reported that acute streptococcal infection results in increased neutrophils infiltrating the lung and the anti-inflammatory agent, a farnesyltransferase inhibitor, was able to reduce this infiltration (Zhang et al. 2012). These results suggest that neutrophils accumulate in the lungs during infection and systemic inflammation.

Neutrophil development

The development of the neutrophil within the bone marrow is divided into six stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band form and mature neutrophil (Fig. 8) (Bainton 1992). The whole process of neutrophil development and maturation in the bone marrow takes around 14 days (da Silva et al. 1994); thereafter, the mature neutrophil is released into the circulation and travels to tissues. The movement of mature neutrophils into the circulation is affected by various factors, such as endotoxin, glucocorticoids and TNF-α (Deinard and Page 1974; Jagels et al. 1995; Mitchell et al.

1982). Neutrophils have function and undergo spontaneous apoptosis within a few days, once they exit the bone marrow (da Silva et al. 1994).

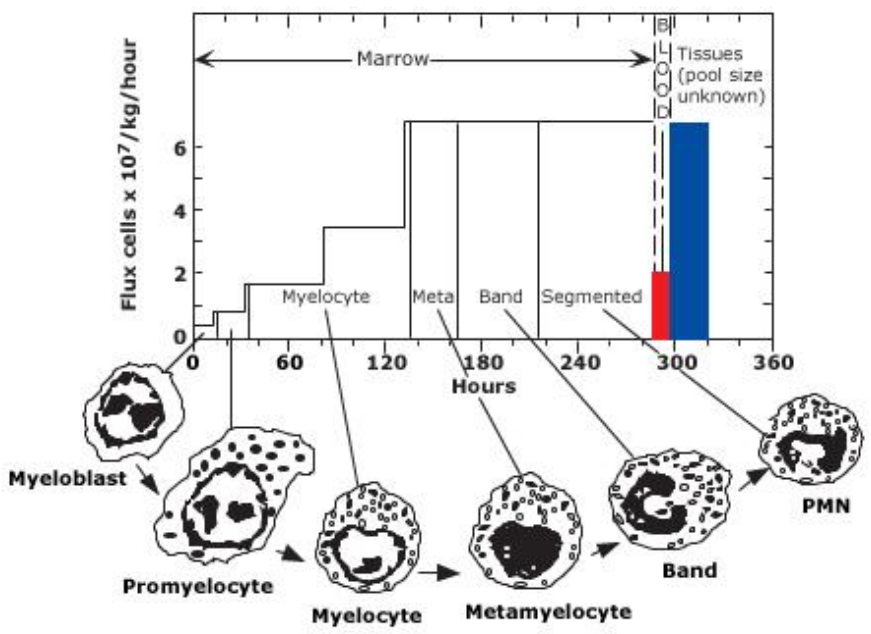

Figure 8 The developmental stages of human neutrophils and their duration. Myeloblasts derive from stem cells, which is followed by two secretory stages: the promyelocyte and the myelocyte. Subsequently, there are non-proliferation and non-secretory stages cells: the metamyelocyte and band cells. From a myeloblast to metamyelocyte (mitotic stage) requires around 7.5 days and from metamyelocyte to mature neutrophils (post mitotic stages) lasts around 6.5 days. Then mature PMN are released into the blood and reach tissues. In the tissues, neutrophils will keep their function around 1-2 days. Red column: neutrophils secreted to blood. Blue column: neutrophils released to the tissues. Figure modified from Bainton (1992).

I.4 Type 2 Diabetes

Diabetes is a metabolic disorder with relative or absolute lack of insulin, a hormone that lowers blood glucose levels. Diabetes therefore results in higher glucose levels in the

body. In Australia, T2D is the second leading cause of health loss in male and the fourth cause of health loss in female (Begg et al. 2008). T2D is characterized by insulin resistance, relative insulin deficiency and hyperglycaemia, whereas Type 1 diabetes is caused by absolute insulin deficiency and hyperglycaemia (Bluestone et al. 2010; Cooke and Plotnick 2008). T2D is often controlled by increasing exercise and dietary modification, although oral medications and insulin injections are often needed, especially in later stages of the disease where the pancreas cannot secrete enough insulin to compensate for the insulin resistance (Solerte et al. 2008). People with type 1 diabetes have to take insulin to control blood glucose levels from the onset of the disease. T2D is a major health burden in modern society and the incidence increases every year(Begg et al. 2008). The major reason for the significant healthcare burden is that people with long term diabetes, tend to have severe complications like renal failure, peripheral neuropathy, diabetic retinopathy and cardiovascular diseases (hypertension, heart attack, stroke and peripheral artery disease) (Begg et al. 2008).

Mechanisms of insulin resistance

Insulin resistance is the key feature of T2D, and there are several metabolic contributors to this aspect of the disease. Firstly, reduced muscle glucagon synthesis is one of the major causes of insulin resistance in T2D patients, as muscle glucagon synthesis is the major source of glucose metabolism (Shulman et al. 1990). Normally, plasma glucose is transferred to the cell and then intracellular glycogen synthesis occurs (Fig. 9). The defective function of GLUTs, especially GLUT4 in T2D, plays an important role in insulin resistance (Shulman 2000) . Defects of hexokinase II is the feature of T2D and this defect results in reduced glycogen synthesis as hexokinase II is an important enzyme for glycogen synthesis (Shulman 2000). It has been demonstrated that the glycogen

synthesis of T2D patients was only 50% of that of normal individuals (Rothman et al. 1995). In addition, it has been demonstrated that plasma FFAs are able to induce insulin resistance by interfering with the transfer and phosphorylation of glucose and thereby reducing GLUT4 function or interfering with the phosphorylation of IRS (Dresner et al. 1999; Shulman 2000). Normally, intracellular insulin signalling starts when insulin binds to the insulin receptor and IRS, and then IRS activates PI3K, which is able to stimulate glucose transfer (Shulman 2000). The interference of GLUT4 reduces the transfer of

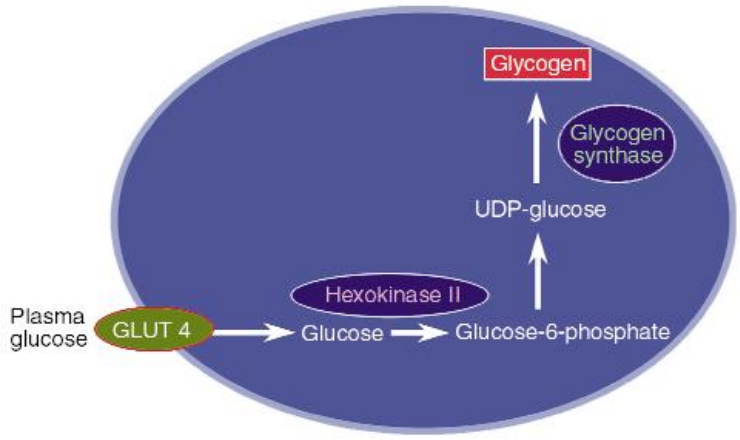

Figure 9 Intracellular glycogen synthesis of glucose metabolism. Plasma glucose is transferred into a cell via GLUT4. With the help of hexokinase II, glucose-6-phosphate and UDP-glucose, intracellular glycogen is synthesized. Adapted from Shulman (2000).

glucose for glycogen metabolism. In T2D, increasing FFA causes increasing intracellular FFA metabolites such as fatty acyl coA, diacylglycerol and ceramide, with phosphorylated IRS thus inactivating PI3K and reducing GLUT4 activation (Dresner et al. 1999; Shulman 2000).

Several studies have shown a relationship between activin A and glucose status, and that activin A can regulate glucose metabolism. For example, activin A can stimulate pancreatic islet β cells to secrete insulin and inhibit pancreatic α cell expression of glucagon, thereby reducing glucose levels (Tanimoto et al. 1991) . However, very few studies have looked into the correlation between activin A and insulin resistance, which is the necessary characteristic of T2D. Apart from T2D, insulin resistance also could be the important characteristic and aetiological factor of some other diseases, such as PCOS and metabolic syndrome, which is also called insulin resistance syndrome (Alberti et al. 2005; Cho et al. 2011). Furthermore, insulin resistance often accompanies other metabolic disorders, such as central obesity, hypertension, dyslipidaemia and atherosclerosis (Cave et al. 2008) In general, insulin resistance is not due to problems with insulin itself, but is a problem with the insulin target tissues with reduced responsiveness to normal circulating concentrations of insulin, so that the pancreas compensates by secreting more insulin (Bierbrauer and Weber-Carstens 2011). Fat and muscle are the insulin target tissues requiring insulin to absorb glucose. Insulin resistance causes reduced absorption of glucose in these target tissues, while insulin resistance in the liver results in reduced capacity for glycogen synthesis. In order to compensate for this reduced responsiveness, the pancreas has to secret more insulin to lower blood glucose and maintain homeostasis, with the result that insulin levels become higher than normal (Rao 2001).

Type 2 diabetes, insulin resistance, obesity and inflammation

Both genetic and environmental factors contribute to the onset of T2D (Schinner et al. 2005). Although there is a strong genetic predisposition, T2D is a generally preventable chronic condition, because lifestyle changes, such as healthy diet and exercise, can reduce some risk factors for T2D, such as high blood pressure, excess weight or obesity

(Lindstrom et al. 2010; Midhet et al. 2010). Obesity and inflammation have together been implicated as important aetiological factors in the development of insulin resistance and the onset of T2D. In fact, inflammation could be the link between obesity and insulin resistance/T2D (Abdin et al. 2010; Shi et al. 2006). Inflammatory M1 macrophages in adipose tissue in people with T2D or obesity are able to secrete elevated levels of inflammatory mediators, such as TNF-α, IL-1β, IL-6 and MCP-1 (Boden 2011). These inflammatory mediators can contribute to insulin resistance by inhibiting insulin signalling in several ways, such as serine-phosphorylation of IRS-1, inducing suppressor of cytokine signalling 3 (SOCS3) and triggering inflammatory signalling via JNK or NF-κB in insulin-target tissues, such as skeletal muscle, liver, and adipose cells (Usui and Tobe 2011). For example, TNF-α activation of the c-JNK-1 (Solinas et al. 2007) and NF-κB signalling pathways (Baud and Karin 2001) can inhibit insulin action in a tissue-specific manner (Wunderlich et al. 2008). The LPS receptor, TLR4, is utilized by free fatty acids, which are elevated in obesity (Boden 2011).These can then induce pro-inflammatory cytokine expression in macrophages, adipocytes and the liver (Shi et al. 2006). Therefore, TLR4 signalling seems to be an integral component of obesity-induced insulin resistance (Shi et al. 2006). It is reasonable to suggest that stimulation of activin A by TLR4 activation might play a similar action as other inflammatory factors.

As mentioned above, JNK-1 is important in obesity-induced insulin resistance and the onset of T2D. In obese mice, JNK-1 is elevated, whereas in JNK knockout obese mice, glucose and insulin levels are significantly lower than in control mice (Hirosumi et al. 2002). The mechanism might be that in obese mice, TNF-α and free fatty acids activate JNK-1 expression, because JNK-1 can reduce the signalling capacity of the insulin receptor through phosphorylation of IRS-1 at Ser307 (Hirosumi et al. 2002). Interfering

with the insulin receptor or IRS-1 will affect activation of PI3K and Akt (a serine/threonine kinase), which can induce glucose uptake through activating GLUT-4 translocation to the plasma membrane (Schinner et al. 2005). Apart from JNK-1, TNF-α is able to activate several other kinases, such as IKK-β, which have similar effects as JNK-1 on the insulin receptor or IRS-1 (Hirosumi et al. 2002). Thereby, impairment of the capacity of IRS-1 to activate downstream PI3K pathway may result in insulin resistance (Aguirre et al. 2000; Gao et al. 2002).

Activins and type 2 diabetes

Activin A is an important inflammatory factor and T2D is regarded as a metabolic disorder with chronic inflammatory features. Thereby, activin A might have a relationship with T2D. Firstly, the release of activin A occurs through activation of TLR4 (Muzio et al. 1998), and TRL4 signalling is also involved in the development of insulin resistance induced by free fatty acids (Shi et al. 2006). It has been established that TLR4 expression, signalling and functional activation were increased in recently diagnosed T2D (Dasu et al. 2010). Apart from TLR4, JNK is another intracellular signalling protein that is common to both T2D and activin A regulation (Funaba et al. 2003). Obesity and inflammation can enhance JNK expression, which can induce insulin resistance and the release of activin A (Funaba et al. 2003; Hirosumi et al. 2002; Muzio et al. 1998). Activins can stimulate the expression of PAI-1 (Hariharan and Pillai 2008), and PAI-1 expression has been implicated in insulin resistance (Hashimoto and Funaba 2011). Secondly, activin A is an important inflammatory factor and elevation of activin A is accompanied by other inflammatory mediators, including TNF- α, while T2D is also associated with increase of inflammatory mediators, such as CRP (Michel et al. 2003). As already mentioned, TNF-α plays an important role in the development of insulin resistance and onset of T2D (Moon

et al. 2010). Thirdly, it is reported that follistatin, along with CRP, is elevated in PCOS, where insulin resistance is an important characteristic and aetiological factor (Chen et al. 2009). Activins can assist the differentiation of pancreatic islet β cells, which enable the islet to produce more insulin, promote glucose uptake and control glucose levels (Hashimoto and Funaba 2011). In addition, glucose-stimulated insulin secretion is increased by activin A in both human and rat islet cultures (Brown and Schneyer 2010; Florio et al. 2000; Totsuka et al. 1988). Moreover, macrophage-induced adipose inflammation could be a bridge between activin A and insulin resistance/T2D. Infiltration of macrophages into adipose causes tissue inflammation and thereby leads to insulin resistance (Hardy et al. 2011; Korner et al. 1997). Conversely, activin A might reduce macrophages inducing inflammation by switching macrophages from the M1 inflammatory phenotype to an M2 anti-inflammatory phenotype (Ogawa et al. 2006; Schenk et al. 2008; Schneider et al. 2000). Furthermore, in patients with acute myocardial infarction, activin A protein levels were higher in patients suffering from abnormal glucose regulation than in patients displaying normal glucose regulation (Moon et al. 2010). Interestingly, it has been found that insulin upregulates the expression of the activin β_B subunit gene in cultured adipocytes, while insulin resistance is characterized by visceral fat deposition, which indicates activin B might play a role in insulin resistance (Hashimoto and Funaba 2011).

Neutrophils and type 2 diabetes

In human studies, insulin was shown to reduce LPS-induced oxidative responses and inflammatory stress, but had no depressive effects on LPS-induced TNF-α and IL-6 elevation (Dandona et al. 2010). Furthermore, it has been demonstrated that insulin treatment is able to attenuate systemic inflammatory responses and modulate immune cell

function (Cuschieri et al. 2008; Walrand et al. 2006). For example, several studies have shown that insulin can bind to receptors on the surface of human neutrophils and attenuate functions such as chemotaxis and phagocytosis (Walrand et al. 2006). These studies suggest that elevated insulin and glucose during T2D could affect neutrophil function. Given the observation that these cells contain and secrete activin A during inflammatory events, this point to a potential linkage between activin, inflammation associated with T2D, and neutrophils.

I.5 Summary

It is known that several cell types and tissues are able to produce activin A and that activin A is elevated during acute inflammation and other inflammatory disorders. However, it is not known what is the major source of circulating activin A under normal conditions, and how it is released during acute inflammation. Following induction by LPS, the first peak of activin A release occurs within one hour, suggesting that acute activin A release comes from pre-stored activin A protein. Furthermore, it is not known whether activin A is involved in T2D, which is a metabolic condition with chronic inflammatory features, but it seems reasonable to suggest that activin A could be elevated in T2D, and play some role in the development or severity of this disease. Finally, since elevated insulin and glucose during T2D might play a role in regulating neutrophil function, the discovery that human neutrophils contain activin A suggests a possible linkage between these cells and the disease, involving activin.

Declaration for Thesis Chapter II

Declaration by candidate

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

Nature of contribution	Extent of contribution (%)
All laboratory work, except some activin, follistatin and tumour necrosis factor-α protein assays. Design of experiments and research planning, analysis of data, drafts of the manuscript and preparation of figures for publication	70

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Yi Chen	Assistance with immunohistochemical studies and tissue collection	10
Wendy Winnalll	Research training and assistance with tissue collection	
David Phillips	Supervision of research, assistance with tissue collection, eiditing and final draft of manuscript	
Mark Hedger	Supervision of research, assistance with tissue collection, editing and final draft of manuscript	

Candidate's Signature	[redacted]	**Date** 12.02.2013

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:

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Chapter II - Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice

Hypothesis: *The early release of activin A following induction of inflammation is due to pre-stored protein and/or rapid synthesis of new protein. Under normal conditions and during inflammation, activin A is released into the circulation from multiple tissues.*

Background

In order to understand the source of activin A, it is important to understand the process of activin A protein synthesis and the release of activin A under normal and inflammatory conditions. The early release of activin A following an acute inflammatory challenge using agents like LPS suggests two possible mechanisms. First, the release could be from pre-existing intracellular stores. Second, the release could be from rapid synthesis of new protein, as appears to be the case for other early inflammatory cytokines, such as TNF-α (Zuckerman et al. 1989). It is known that activin A is produced *de novo* by a number of cell types following inflammatory stimulation, including monocyte/macrophages (Eräämaa et al. 1992; Sugama et al. 2007), dendritic cells (Robson et al. 2008), Th2 cells (Ogawa et al. 2006), endothelial cells (Wilson et al. 2006) and bone marrow stromal cells (Uchimaru et al. 1995).

The process of protein synthesis involves two main steps. First, transcription occurs in the nucleus where mRNA is copied from DNA. Second, translation occurs in the cytoplasm where mRNA encodes new protein synthesis (Agirrezabala and Frank 2010). There are

reagents that can specifically block these two steps. Actinomycin D is primarily used as an investigative tool in cell biology to inhibit transcription. It does this by binding DNA at the transcription initiation complex and preventing elongation of growing RNA chains (Sobell 1985). Cycloheximide is a eukaryotic protein synthesis inhibitor, which exerts its effect by interfering with the translocation step in protein synthesis, that is, the movement of tRNA on the ribosome (Schneider-Poetsch et al. 2010). Actinomycin D and cycloheximide were used to interfere with the synthesis of activin A in the LPS-induced inflammation model in adult mice. In order to investigate the actual tissue source of activin A release, a broad range of tissues were collected from untreated control mice and LPS-treated mice, and both mRNA and protein were measured. As follistatin is the major regulator of activin action in the circulation and tissues, production of this protein was also measured in these mice.

Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice

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Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice

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Wu H, Chen Y, Winnall WR, Phillips DJ, Hedger MP. Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice. *Am J Physiol Regul Integr Comp Physiol* 303: R665–R675, 2012. First published August 1, 2012; doi:10.1152/ajpregu.00478.2011.—Activin A, a member of the transforming growth factor-β family, increases in the circulation within 1 h after administration of bacterial LPS. To clarify the origins of this rapid increase, the distribution of activin A and its binding protein, follistatin, and their production following LPS treatment, were assessed in adult male mice. In untreated mice, activin A was detectable in all 23 tissues examined, with highest mRNA expression (as measured by quantitative RT-PCR) was found in the liver, and the largest concentration of activin A protein (by ELISA) was found in the bone marrow. Likewise, follistatin mRNA and protein were present in all tissues, with highest expression in the vas deferens. Activin A and follistatin mRNA did not increase significantly in any tissue within the first hour after LPS, but activin A protein decreased by 35% in the bone marrow and increased 5-fold in the lung. No significant changes were observed in any other tissue. Activin A reached a peak in the circulation 1 h following LPS, and then declined. Cycloheximide, an inhibitor of protein translation, reduced this increase of activin A by more than 50%. Actinomycin D, an inhibitor of mRNA transcription, had no effect. Circulating follistatin did not increase until 4 h after LPS and was not affected by either inhibitor. These data indicate that the rapid increase in circulating activin A during LPS-induced inflammation is regulated at the posttranscriptional level, apparently from newly translated and stored protein, and implicate bone marrow-derived cells, and, in particular, neutrophils, as a significant source of this preformed activin A.

activin a; follistatin; tumor necrosis factor-α; lipopolysaccharide; inflammation

ACTIVIN A IS A DISULFIDE-LINKED homodimeric protein and a member of the transforming growth factor-β (TGF-β) superfamily of growth and development cytokines (10). It is widely produced and distributed in the body and has been shown to play critical roles in embryo development, liver function, reproduction, and immunoregulation. Activin A is also involved in inflammation, and it has been implicated in the progression of many inflammatory diseases, including rheumatoid arthritis, asthma, and meningitis (10). Serum and tissue concentrations of activin A increase during infection and inflammation, and elevated circulating levels of activin A are predictive of death in human and experimental models of septicemia (13, 20). Follistatin, which is structurally unrelated to the TGF-β superfamily, binds activin A with high affinity and blocks the ability of activin A to bind to its receptor and initiate downstream signaling (36). Animal studies show that injection of exogenous follistatin can improve survival following septic shock (13) and reduces the severity of inflammation and subsequent damage in diseases, like colitis and allergic asthma, and in experimental lung and liver damage models (2, 7, 9, 27).

In animal models, injection of the bacterial cell wall component with LPS, causes acute systemic inflammation, which results in an increase of activin A in the bloodstream within 1 h, along with the key inflammatory cytokines, TNF-α, IL-1β, and IL-6 (12, 13). Stimulation of these cytokines involves activation of the Toll-like receptor 4 by LPS in responsive cells and signaling through the inflammatory adaptor protein, MyD88 (16). In fact, the rapid increase in activin A in the circulation coincides with, and may even precede, the release of TNF-α, which is one of the most rapidly released inflammatory regulators (12). Follistatin increases in the circulation several hours later, possibly in response to stimulation by activin A itself (13), although it may also be directly regulated by LPS, TNF-α, or IL-1β (15, 21, 29).

In mice that had been pretreated with exogenous follistatin 30 min prior to the injection of LPS, there was a substantial reduction in TNF-α and IL-6 production, and a significant delay in the release of IL-1β (13). This effect of blocking activin A by follistatin was consistent with studies that have shown that activin A stimulates production of these cytokines, and other proinflammatory genes, in human, rat, and mouse mononuclear phagocytes in vitro (22, 23, 42). These data indicate a crucial role for activin A, and, consequently, for endogenous follistatin, in controlling the severity of acute inflammation and its subsequent actions.

Despite the evidence of a critical involvement of activin A in acute inflammation, the source and regulation of activin A following LPS administration remain unknown. The very rapid release of activin A suggests two possible mechanisms. First, the release could be from rapid synthesis of new protein, as appears to be the case for TNF-α (46). It is already well known that activin A is produced de novo by a number of cell types following inflammatory stimulation, including mononuclear phagocytes and dendritic cells (8, 30, 37), endothelial cells, (40) and several bone marrow cell types (35, 44). Alternatively, activin A could be released from preexisting intracellular stores. Several studies have shown that the activin A subunit (β_A-subunit) gene is widely expressed even under normal conditions (19, 34, 38), but there has been no comprehensive quantitative examination of this distribution, or the response to LPS administration in vivo.

In this study, we examined several candidate tissue sources of activin A and follistatin and the regulation of their synthesis during acute inflammation induced by an LPS challenge. Inhibitors of protein synthesis (cycloheximide) and mRNA synthesis (actinomycin D) were used to establish whether the acute release of activin A after challenge by LPS is due to release of

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stored protein or de novo synthesis. These data will be able to inform future studies on the role of activin A in inflammation, as well as the potential for follistatin to act as a specific therapeutic agent for controlling activin A actions in inflammation and its consequences.

MATERIALS AND METHODS

Animals

Adult male mice (8–10 wk old; 18–22 g) of the C57BL6/J strain were used in all studies. Procedures involving animals were conducted in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care of Scientific Purposes (1997) and were approved by the Monash Medical Centre Animal Ethics Committee.

Reagents

Solutions of actinomycin D, cycloheximide, and LPS (*E. coli* serotype 0127:B8) were freshly prepared at a final concentration of 1 mg/ml in 0.9% saline on the day of the experiment. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Tissue Distribution Study

Experiment 1. Five control mice were injected (i.p.) with saline alone (0.1 ml), and 5 mice were injected with 100 μg LPS in an equivalent volume of saline. Mice were euthanized 1 h after injection, and the following tissues were collected: flank skin, lymph nodes (pool of axillary, mediastenial, cervical, brachial, mesenteric, and inguinal nodes), hind-limb skeletal muscle, brain cortex, eye, lung, heart, thymus, liver, spleen, pancreas, small intestine, adrenal, kidney, bladder, vas deferens, testis, epididymis, seminal vesicle, ventral prostate, thyroid, and femoral bone. Bone marrow-derived cells were collected by syringe from the femurs by injection of PBS and centrifuged at 5,000 *g* for 5 min at 4°C. Blood was obtained by cardiac puncture and stored at 4°C overnight. The following day, the serum was collected by centrifugation at 5,000 *g* for 10 min at room temperature, then transferred to a fresh tube, and stored at [minus]20°C until assay. Tissues were stored at [minus]80°C before being processed for protein assays and mRNA measurement or were immersion-fixed in formalin or Bouin's fixative (for testes only), processed on a Leica ASP300 vacuum processor (Leica, Solms, Germany) for embedding in paraffin, and sectioned (5 μm) using a Leica RM2135 microtome onto Superfrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany). Whole bone samples were decalcified overnight in 20% formic acid prior to processing. Slides for staining were incubated in a 60°C oven for 20 min and stained with Harris hematoxylin and 1% aqueous eosin (Amber Scientific, Midvale, Western Australia) before coverslipping with Neo-mount (Merck, Darmstadt, Germany).

Experiment 2. Seven control mice were injected intraperitoneally with saline alone (0.1 ml), and 7 mice were injected with 100 μg ip LPS in an equivalent volume of saline. Mice were euthanized 1 h after injection, and the bone marrow-derived cells and liver were collected, as in *experiment 1*. Blood obtained by cardiac puncture was collected into 1.5-ml Eppendorf tubes containing 15 U heparin and centrifuged at 3,000 *g* for 10 min at room temperature. The plasma fraction and leukocytes were collected by careful aspiration. All tissues and cells were stored at [minus]80°C until processed for protein assays and mRNA measurement.

Regulation of Activin A and Follistatin by LPS

Experiment 3. Mice were divided into the following treatment groups: injection of 100 μg ip LPS alone (6 mice/time point), injection of cycloheximide (5 mg/kg ip) 60 min prior to an injection of 100 μg ip LPS (6 mice/time point), or 0.1 ml saline alone (6 mice/time point); injection of actinomycin D (5 mg/kg ip), 60 min prior to an injection of 100 μg LPS (4 mice/time point), or 0.1 ml saline alone (4 mice/time point). The concentrations of cycloheximide and actinomycin D used were based on those used by Zhang et al. (45). Blood and liver samples were collected at 0, 0.5, 1, 2, 3, 5, and 7 h after injection of LPS or saline (with the 0-h group acting as control), as described in *experiment 1*. Mice were monitored throughout the experimental procedure, and mice exhibiting evidence of severe distress were euthanized. As a result, in the actinomycin D with LPS treatment groups, two of the mice at 5 h and all of the mice at 7 h were removed from the experiment.

Experiment 4. Mice were injected intraperitoneally with saline alone or 100 μg ip LPS, with or without a prior injection of cycloheximide (5 mg/kg) 60 min earlier (6 mice/treatment group). Bone marrow-derived cells and serum were collected and processed for assay, as described in *experiment 1*.

Tissue Extraction for Total Protein Quantification

Tissue and cell samples were homogenized in PBS containing protease inhibitors (EMD Biosciences, La Jolla, CA). After homogenization, samples were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatants were collected. Total protein was measured in the extracts using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), as per the manufacturer's instruction. The sensitivity of the assay was 1 μg/ml.

Immunoassays

Activin A was measured by a two-site ELISA (Oxford Bioinnovations, Cherwell, Oxfordshire, UK), using human recombinant activin A as a standard (17). This assay measures both free and follistatin-bound activin A dimers and has no significant cross reaction with other isoforms of activin or TGF-β. The mean assay sensitivity was 12 pg/ml. The mean intra-assay and inter-assay coefficients of variation (CVs) were 5.0% and 6.7%, respectively.

Follistatin was measured by RIA, using human recombinant follistatin 288 as standard and tracer, as described previously (24). This assay measures total follistatin. The mean assay sensitivity was 0.85 ng/ml. The mean intra-assay and inter-assay CVs were 12.1% and 5.0%, respectively.

TNF-α was measured by a Quantikine mouse TNF-α ELISA (R&D Systems, Minneapolis, MN), using mouse recombinant TNF-α as a standard (28). The sensitivity of the assay was 12 pg/ml. The mean intra-assay and inter-assay CVs were 6.8% and 8.4%, respectively.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

Tissue samples were processed and assessed for mRNA expression levels using established methods, which have been described in detail previously (6, 41). All tissues for mRNA measurements were stored at [minus]80°C. Total RNA was extracted and purified from homogenized tissue by lysis in 1 ml TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. After extraction, RNA solutions were treated with DNAse to remove genomic DNA using a DNA-free kit (Ambion, Austin, TX). All data in each experiment were determined from samples of RNA extracted at exactly the same time under identical conditions to eliminate variation in mRNA extraction efficiency as a result of procedural differences.

Oligo-dT-primed cDNA was synthesized from 1 μg of RNA using the Superscript kit (Invitrogen). The amount of mRNA used to make cDNA was equalized for all tissues to minimize errors due to differences arising from small amounts of starting mRNA. Sample cDNAs were diluted 1:10 in diethylpyrocarbonate-treated water immediately prior to PCR reactions, and 2 μl of this diluted cDNA was added to each reaction. For each tissue cDNA, a nonreverse-transcribed control was produced to act as an internal negative control. A phagemid-

derived RNA "spike" was added to each reaction as an external standard control, as previously described (6, 41).

Gene expression was measured by quantitative PCR (qPCR) using a Bio-Rad iQ5 system (Bio-Rad, Hercules, CA) and the Fast-Start DNA Master SYBR-Green system (Roche, Mannheim, Germany). The primers for the activin β_A-subunit were 5′-TGGAGTGTGATGGCAAGGTC-3′ (forward) and 5′-AGCCACACTCCTCCACAATC-3′ (reverse); for total follistatin (i.e., to detect both *Fst288* and *Fst315* variant mRNA), the primers were 5′-CCACTTGTGTGGTGGATCAG-3′ (forward) and 5′-AGCTTCCTTCATGGCACACT-3′ (reverse); for IL-1β, the primers were 5′-CCAGGATGAGGACCCAAGCA-3′ (forward) and 5′-CCCGACCATTGCTGTTTCC-3′ (reverse); and for TNF-α, the primers were 5′-TCATCAGTTCTATGGCCCAGAC-3′ (forward) and 5′-CCCTTGAAGAGAACCTGGGAGT-3′ (reverse). In addition to a blank negative control and nonreverse-transcribed cDNA negative control, the external standard RNA spike was used to normalize reaction efficiency, to avoid the variability of other housekeeping genes from different tissues (6). This spike was detected at the same level in each tissue, and all data were analyzed by relative quantitation to the RNA spike, using the $2^{-\Delta\Delta Ct}$ method. The efficiency of each reaction was between 87% and 110%, and the patterns of gene expression were highly reproducible. Data are presented as relative expression compared with liver as the reference tissue.

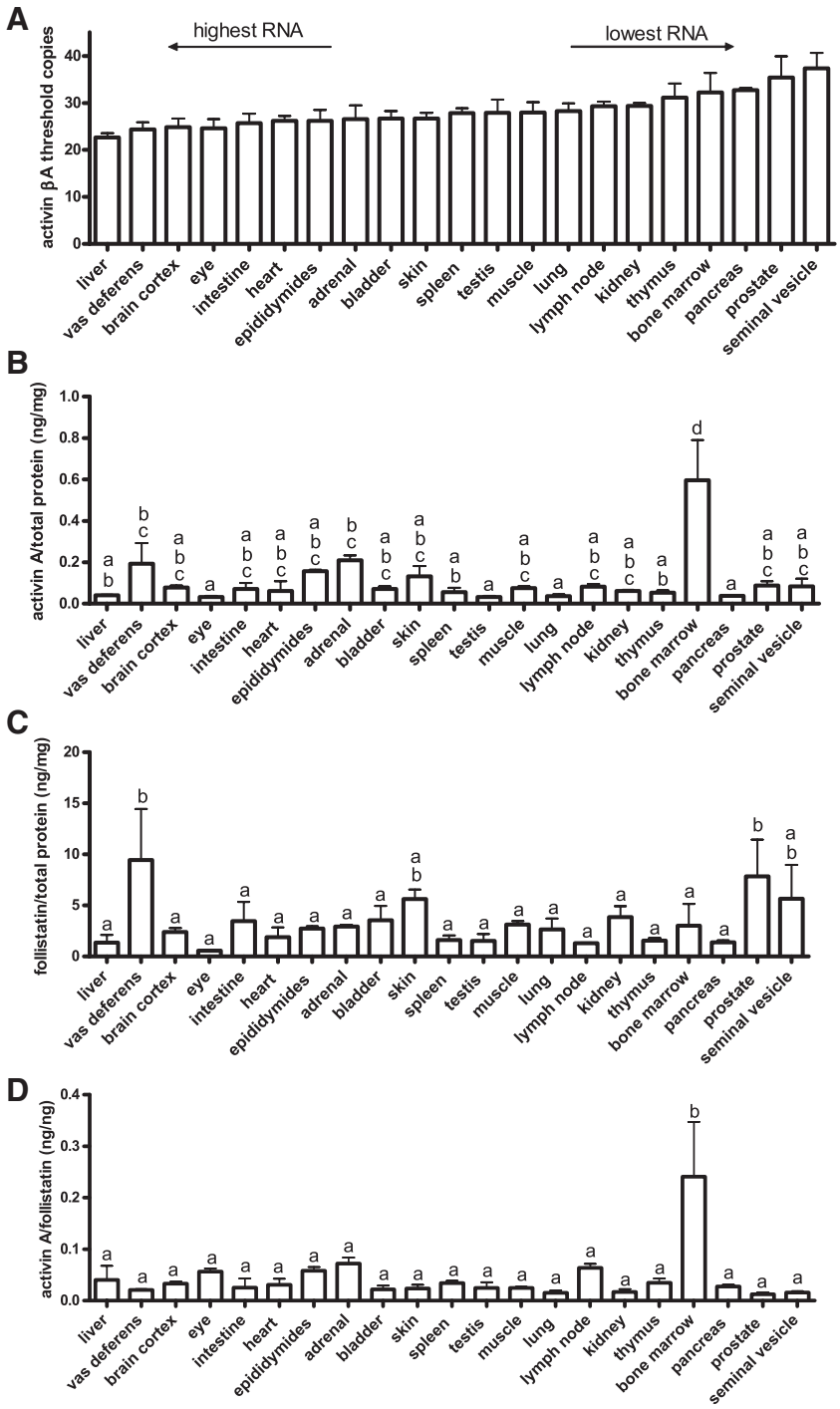

Fig. 1. Tissue distribution of activin β_A-subunit mRNA, activin A, follistatin, and activin A:follistatin ratio in untreated mice. *A*: mean threshold copy number for activin A mRNA measured by quantitative PCR (mean ± SD; $n = 5$ samples) in multiple runs. Threshold copy number is inversely proportional to mRNA concentration in the tissue, which means that the highest threshold copy number indicates the lowest amount of mRNA. *B*: activin A levels in tissues relative to total protein (means ± SD; $n = 3$). *C*: follistatin levels in tissues relative to total protein (mean ± SD; $n = 3$). *D*: ratio of activin A to follistatin in tissues, as an indicator of relative levels of free activin A. Note that the ratio value is based on immunoactivity estimates, and not absolute mass values, for activin A and follistatin. [a,b,c,d]Values with letters that differ are significantly different.

Immunohistochemistry for Activin A

Activin A immunohistochemistry was performed on cytospin preparations of bone marrow-derived cells and 5-μm paraffin sections of lung tissue and decalcified whole bone, as previously described (4, 25). A mouse monoclonal antibody (E4) directed toward the β_A subunit of activin A was used as a primary antibody (17). An isotype-matched mouse antibody (IgG2b) was used as a negative control.

Statistical Analyses

One-way and two-way ANOVA were used to determine significant differences between groups, following suitable transformation to normalize the data and equalize variance, in conjunction with a Dunnett's multiple comparisons of means test (Prism 5, GraphPad Software, San Diego, CA). All values are presented as mean ± SD.

RESULTS

Tissue Distribution of Activin A and Follistatin in Adult Male Mice

Activin β_A-subunit mRNA was detectable in every tissue examined by qPCR in normal control mice (Fig. 1*A*). It was not possible to assay all tissues in a single qPCR run for direct quantitative comparison; however, multiple qPCR analyses of the mean crossing threshold (Ct) for each tissue, which is inversely related to the amount of mRNA present, indicated that the liver had the highest β_A-subunit mRNA expression (i.e., lowest threshold crossing point; 22.6 ± 0.9; $n = 5$), while the seminal vesicles and prostate had the lowest β_A-subunit mRNA expression (i.e., highest threshold crossing points; 35.4 ± 4.5 and 37.3 ± 3.2, respectively) (Fig. 1*A*). The thyroid

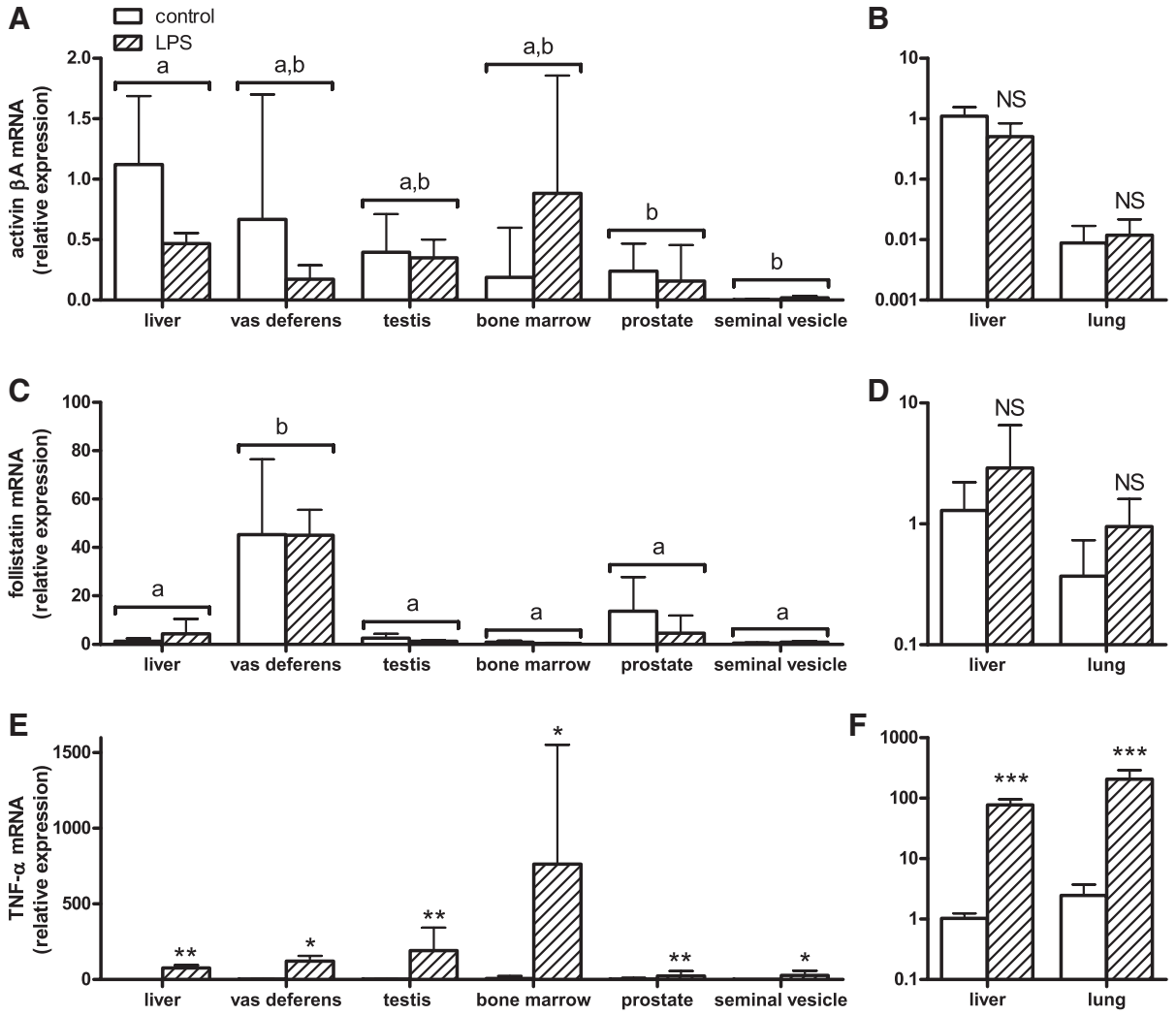

Fig. 2. Comparison of mRNA expression levels in liver, vas deferens, testis, bone marrow, prostate, seminal vesicles, and lung of control mice and mice injected with LPS 1 h previously. *A* and *B*: activin β_A-subunit. *C* and *D*: follistatin. *E* and *F*: TNF-α: left panels are representative qPCR comparisons incorporating the liver (highest activin A mRNA expression) and seminal vesicles (lowest activin A mRNA expression), the male reproductive tract tissues, and bone marrow-derived cells, measured in a single qPCR run (*y*-axis is linear). Right panels are a direct qPCR comparison of liver and lung, measured in a single qPCR run (*y*-axis is log10). All data are presented are means ± SD; $n = 5$ samples, except prostate ($n = 7$). There was no significant difference between control and LPS treatment expression levels for either activin β_A-subunit or follistatin, and tissues with same letter superscript were not significantly different by two-way ANOVA. NS, not significantly different from control at $P > 0.05$. Comparisons for TNF-α between control and LPS-treatment expression levels: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with equivalent tissue control group.

and total blood leukocyte preparations (not included in Fig. 1) also had very high mean threshold crossing points (34.5 ± 1.7 and 33.4 ± 2.9, respectively). Liver, as the tissue with the highest expression of β_A-subunit mRNA, was used as the reference standard tissue for all subsequent analyses.

Comparing relative mRNA concentrations in a selection of tissues during a single qPCR run established that there was a 180-fold difference in β_A-subunit gene expression between the liver (highest expression, lowest Ct) and the seminal vesicles (lowest expression, highest Ct) in control mice and that all other tissue expression levels fell between these limits (Fig. 2, *A* and *B*). In spite of these large differences in β_A-subunit mRNA expression, there was no significant difference between activin A protein concentrations in most tissue extracts, with the exception of bone marrow-derived cells, which contained activin A at levels at least 10-fold higher than the majority of other tissues in control animals (Fig. 1*B*). Levels of activin A in thyroid and total blood leukocyte samples were below the limit of assay detection (<0.005 ng/mg protein).

Follistatin mRNA was observed in all tissues but displayed lower levels and less between-tissue variation of expression compared with the activin β_A-subunit mRNA (Ct range: 27.7 −38.6). In control mice, the vasa deferens showed the highest follistatin mRNA expression, which was at least 10 to 15 times higher than any other tissue examined (Fig. 2, *C* and *D*). The concentration of follistatin protein was similar in most tissues, with the exception of the vas deferens and prostate, which had significantly higher levels of follistatin protein than all other tissues, with the exception of the skin and seminal vesicle (Fig. 1*C*). When activin A levels were expressed as a ratio of activin A:follistatin, as an index of activin A bioavailability in the tissue, bone marrow-derived cell activin A was still seen to be considerably higher (~10-fold) than in all other tissues (Fig. 1*D*).

These data demonstrated that, in spite of a wide range of β_A-subunit mRNA expression, activin A protein was relatively evenly distributed in most tissues, together with its binding protein, follistatin. The clear exception was the bone marrow-derived cells, where both total activin A and the activin A:follistatin ratio were much higher than in all other tissues, even though these cells did not display exceptionally high β_A-subunit mRNA expression.

Tissue Responses to LPS Administration

There was no significant increase in either activin β_A-subunit mRNA (Fig. 2, *A* and *B*) or follistatin mRNA (Fig. 2, *C* and *D*) 1 h following LPS administration, in any of the tissues or isolated cell preparations (total blood leukocytes and bone marrow-derived cells) examined. In contrast, after 1 h of LPS stimulation, expression of TNF-α (Fig. 2, *E* and *F*) and IL-1β (data not shown), mRNA was significantly increased in all tissues and cell preparations.

One hour after LPS administration, the lung samples showed a five-fold increase in activin A protein concentration and the activin A:follistatin ratio, while the bone marrow cells showed a 35% decrease in content (Fig. 3, *A* and *C*). There was no change in activin A concentrations 1 h after LPS treatment in any other tissue or in total blood leukocytes. Follistatin protein was slightly increased in lymph node, but not in any other tissue, 1 h after LPS treatment (Fig. 3*B*).

These data indicate levels of activin A and follistatin do not change in most tissues within 1 h of LPS administration, consistent with the lack of change in mRNA; however, there is a significant decline in activin A in the bone marrow and a corresponding increase in the lung. This suggests that there may be a net transfer of activin A from the bone marrow to the lungs within 1 h after LPS treatment.

Regulation of Activin A and Follistatin in Serum

Following LPS administration, serum activin A increased rapidly in the circulation and reached a peak concentration at 1 h, then gradually declined, but was still elevated 7 h later

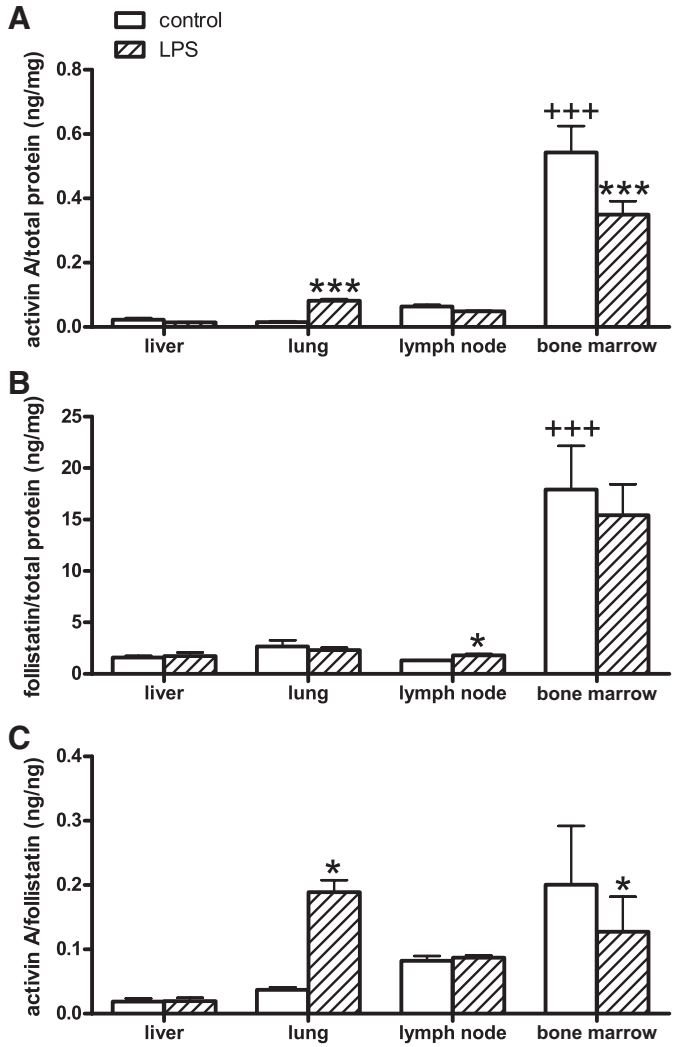

Fig. 3. Comparison of activin A and follistatin protein in liver, lung, lymph nodes, and bone marrow of control mice and mice injected with LPS 1 h previously. *A*: activin A. *B*: follistatin. *C*: ratio of activin A to follistatin in tissues, as an indicator of relative levels of free activin A. Liver is shown as control tissue, together with lung, lymph nodes, and bone-marrow-derived cells, which were the only tissues examined that showed any significant change following LPS treatment. All data presented are means ± SD; $n = 10$ samples (liver and bone marrow) or 3 (lung and lymph nodes). $^*P < 0.05$, $^{***}P < 0.001$ compared with equivalent control group; all other comparisons were not significantly different ($P > 0.05$). $+++P < 0.001$, compared with liver control group; all other comparisons were not significantly different.

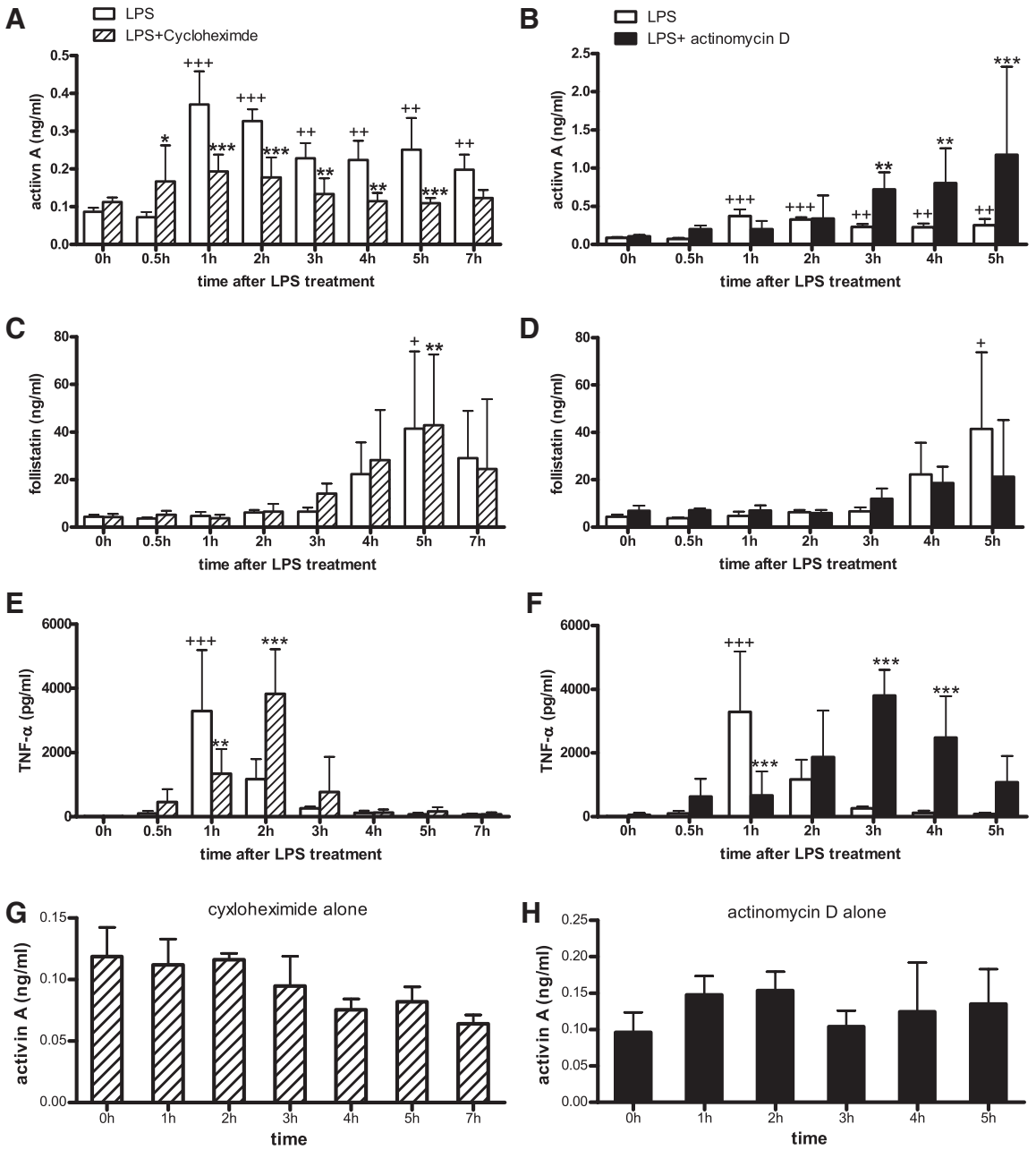

Fig. 4. Time course of serum activin A, follistatin, and TNF-α in mice treated with LPS alone, cycloheximide, or actinomycin D, together with LPS and cycloheximide or actinomycin D alone. *A* and *B*: activin A. *C* and *D*: follistatin. *E* and *F*: TNF-α. *G* and *H*: activin A following treatment with cycloheximide or actinomycin D alone. All data presented are mean ± SD; $n = 6$ samples, except for actinomycin D treatment groups ($n = 4$), and cycloheximide alone ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with equivalent LPS only group; all other comparisons were not significantly different $P > 0.05$. +$P < 0.05$, ++$P < 0.01$, +++P <0.001, compared with 0 h control group; all other comparisons were not significantly different $P > 0.05$.

(Fig. 4, *A* and *B*). Follistatin also increased in the circulation after LPS, but this occurred much later, reaching a peak concentration at 5 h (Fig. 4, *C* and *D*). The release of TNF-α into the circulation coincided with the release of activin A but declined much more rapidly (Fig. 4, *E* and *F*).

Injection of the protein synthesis inhibitor, cycloheximide (5 mg/kg), 1 h before LPS reduced activin A concentrations in the circulation by more than 50% (Fig. 4*A*), had no effect on follistatin (Fig. 4*C*), and delayed the peak release of TNF-α by 1 h without changing the apparent size of the peak (Fig. 4*E*). Administration of cycloheximide alone to mice was followed by a declining trend (i.e., not statistically significant) in activin A concentrations in the blood (Fig. 4*G*) and had no effect on either follistatin or TNF-α levels over 7 h (data not shown). Cycloheximide had no additional effect on the decline in activin A in bone marrow-derived cells 1 h after LPS treatment but reduced activin A concentrations by more than 60% in bone marrow-derived cells from control mice (Fig. 5).

Injection of actinomycin D (5 mg/kg), an mRNA transcription inhibitor, had no significant effect on the initial rise in activin A after LPS treatment but caused a gradual increase in activin A in the blood 3 h after LPS treatment (Fig. 4*B*).

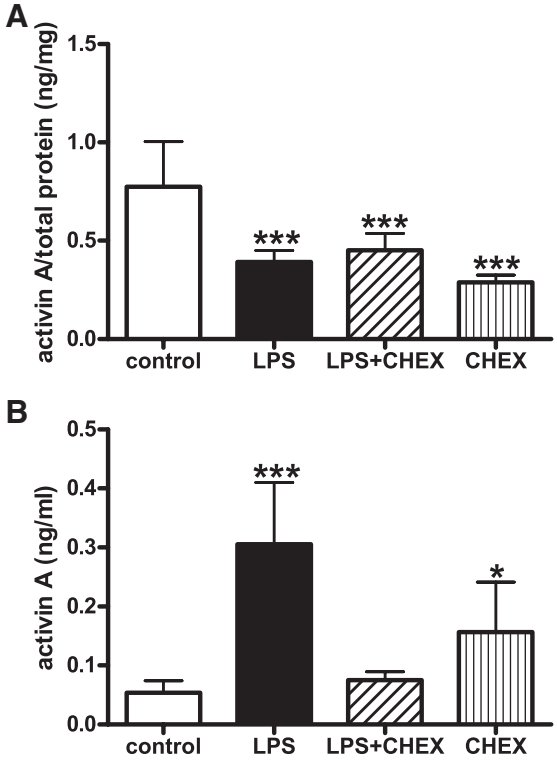

Fig. 5. Activin A in bone marrow-derived cells and serum of mice treated with normal saline, LPS, cycloheximide (CHEX) alone, and together with LPS. *A*: bone marrow-derived cells. *B*: serum. All data presented are mean ± SD; $n = 7$ samples. $^*P < 0.05$, $^{***}P < 0.001$ compared with equivalent normal saline group; all other comparisons were not significantly different $P > 0.05$.

Actinomycin D had no effect on follistatin release after LPS (Fig. 4*D*) but caused both a delay and an increase in the duration of the peak of TNF-α after LPS (Fig. 4*F*). Administration of actinomycin D alone to mice had no effect on activin A (Fig. 4*H*), follistatin, or TNF-α (data not shown) within 5 h.

Although activin β_A-subunit gene expression in the liver was not stimulated in the first hour after LPS administration, there was a significant decline in expression in the liver (tissue with highest level of activin A mRNA) by 4 h after LPS treatment (Fig. 6*A*). In contrast with activin A, follistatin mRNA increased gradually after LPS treatment (Fig. 6*B*), and TNF-α mRNA expression was rapidly induced by LPS (Fig. 6*C*).

Cycloheximide caused a transient increase in the expression of all three genes following LPS treatment (Fig. 6, *A–C*). Actinomycin D had no effect on activin β_A-subunit expression but blocked the induction of follistatin and TNF-α mRNA by LPS (Fig. 6, *B* and *C*).

The fact that the rapid release of activin A into the blood following LPS administration could be substantially blocked by cotreatment with the protein synthesis inhibitor (cycloheximide), but not by an inhibitor of mRNA transcription (actinomycin D), was consistent with the observation that LPS had no acute stimulatory effect on activin β_A-subunit mRNA expression in any tissue. Altogether, these data indicate that activin A levels in the serum are regulated at the posttranscriptional level under both basal and LPS-stimulation conditions. On the other hand, both follistatin and TNF-α appear to be primarily regulated by LPS at the transcriptional level, under the same conditions, but over a different time frame.

Immunohistochemical Localization of Activin A in Lung and Bone Marrow

Immunohistochemical examination of the bone marrow cells collected from control mice indicated that the only cells containing significant activin A protein were the neutrophil precursors (band neutrophils, segmented neutrophils, and ring-form myelocytes) (Fig. 7*A*). Activin A tended to be perinuclear in its distribution in these isolated cells. Following LPS treatment, the number of neutrophil precursors in bone marrow samples declined from 46 ± 3% of total cells (in control mice, $n = 4$) to 19 ± 2% of total cells (in LPS-treated mice, $n = 4$), although many mononuclear cells in these samples now contained activin A protein as well (Fig. 7*B*). In the lungs from control mice, activin A was present in the broncheolar and alveolar epithelial cells and alveolar macrophages (Fig. 7*D*). After LPS treatment, this pattern of staining persisted, but

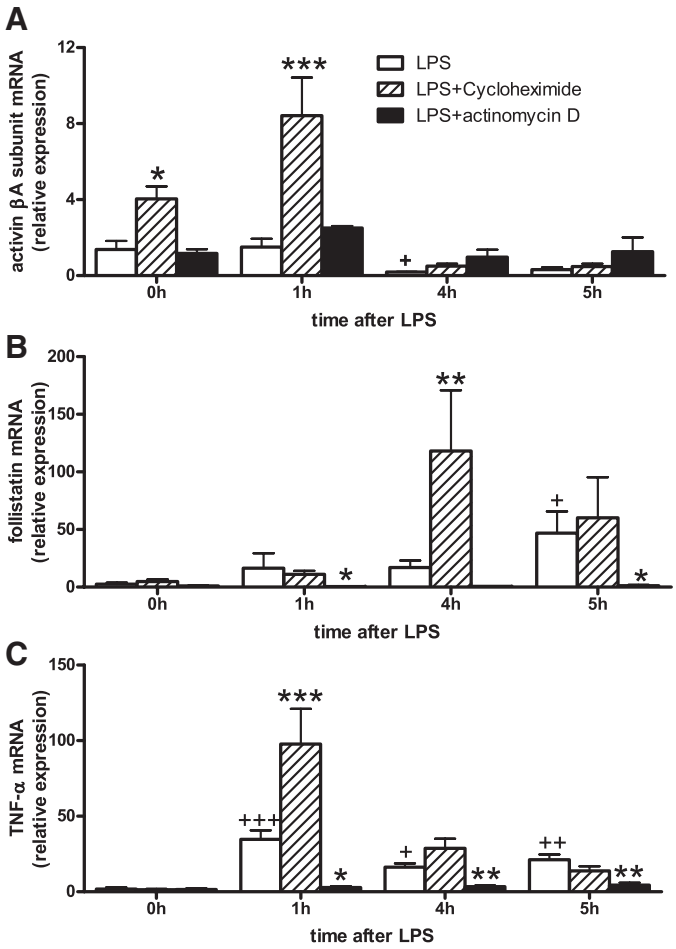

Fig. 6. Activin A, follistatin, and TNF-α mRNA expression in liver from mice treated with LPS alone, or LPS together with cycloheximide or actinomycin D, measured in a single qPCR run. *A*: activin β_A-subunit. *B*: follistatin. *C*: TNF-α. All data are presented as mean ± SD; $n = 6$ samples, except for actinomycin D treatment groups ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with equivalent LPS-only group; all other comparisons were not significantly different ($P > 0.05$). $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$, compared with 0 h control group; all other comparisons were not significantly different ($P > 0.05$).

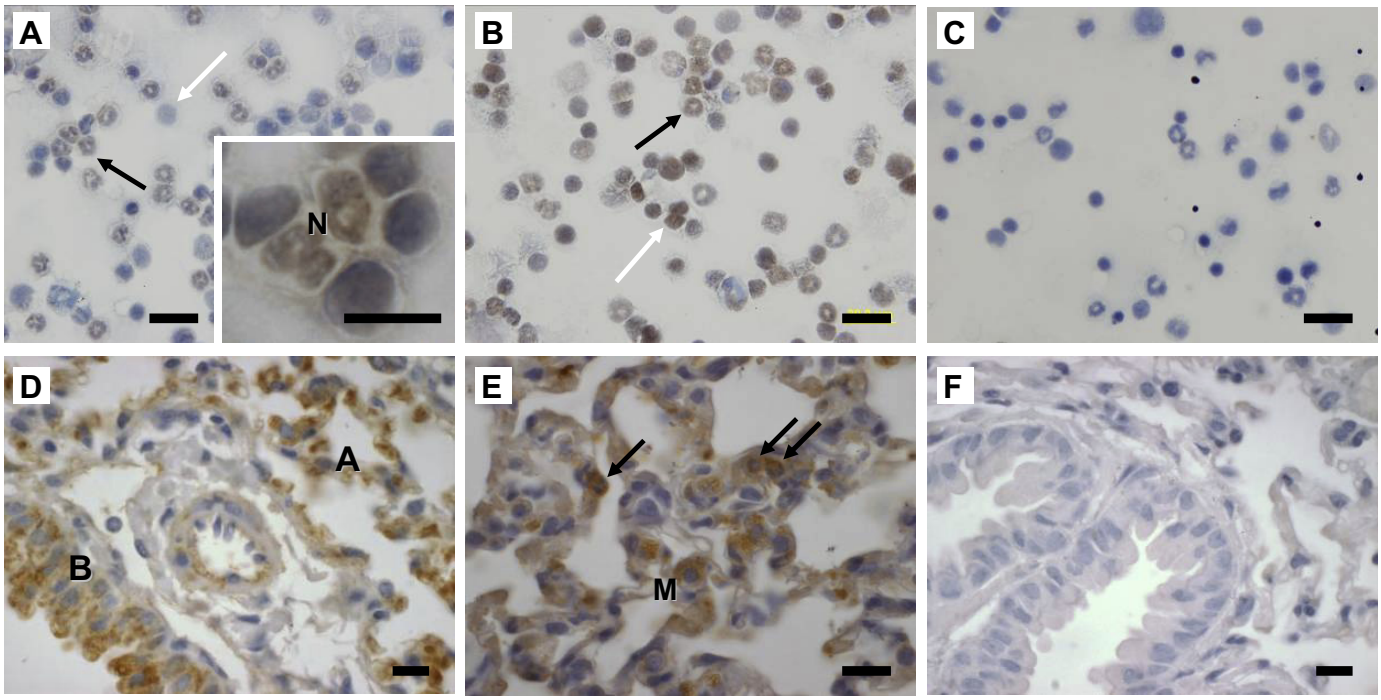

Fig. 7. Immunohistochemical localization of activin A in bone marrow-derived cell cytospin preparations and lung sections of control mice and mice injected with LPS 1 h previously. *A*: neutrophil precursors (N) were the only cell type among bone marrow-derived cells from control mice containing significant activin A protein (black arrows in all panels). White arrows indicate mononuclear cells (monocyte and erythroid precursors). *B*: after LPS treatment, the number of neutrophil precursors in bone marrow-derived cells was considerably reduced, and some mononuclear cells now contained activin A protein (white arrow). *C*: negative control for bone marrow-derived cells. *D*: activin A immunoreactivity was localized to broncheolar (B) and alveolar (A) epithelial cells and alveolar macrophages (not shown). Neutrophils were rarely observed in the normal lung. *E*: after LPS treatment, airway epithelial and alveolar macrophages (M) continued to contain activin A, but activin A-containing neutrophils (black arrows) were frequently observed in the lung as well. *F*: negative control for lung. Scale bar: ~20 μm.

numerous neutrophils accumulated in the lungs, and these cells were also strongly activin A positive (Fig. 7*E*).

In sections of intact bone from normal mice, approximately half of the cells in the bone marrow were positive for activin A (Fig. 8*A*). The majority of these activin A-containing cells possessed the distinctive polymorphonuclear morphology of bone marrow neutrophil precursors (band neutrophils, segmented neutrophils, and some ring-form myelocytes), but labeled megakaryocytes, osteoclasts, chondrocytes, endothelial cells, barrier cells, and bone-lining cells were also observed. By contrast, the majority of mononuclear cells within the bone marrow, which includes the mononuclear myelocytes, rubricytes, and lymphocytes, were activin A-negative, although small numbers of labeled mononuclear cells were observed. After treatment with LPS, the number of activin A-positive cells in the bone marrow declined dramatically (to around 20% of total cells), and the majority of the remaining activin A-containing cells were of mononuclear cell morphology (Fig. 8*B*). These observations are indicative of a loss of activin A from the bone marrow due to extravasation of neutrophils containing prestored activin A after LPS treatment but are also be consistent with release of activin A stored in other bone cell types as well.

Altogether, these data suggest that a significant proportion of the increase in activin A protein levels in the lungs following LPS administration may be due to translocation of neutrophils containing preformed activin A from the bone marrow. Accordingly, the bone marrow shows a reduction in activin A content concurrent with the reduction in neutrophil precursors in this tissue.

DISCUSSION

The major aim of this study was to investigate the source and regulation of activin A and its binding protein, follistatin, during acute LPS-induced inflammation. The results of the distribution of activin A mRNA and protein across 23 tissues and cell preparations in the adult male mouse show that, under normal conditions, the liver has the highest mRNA expression, but contains relatively little activin A protein, while bone marrow cells have the highest endogenous protein levels, but relatively low mRNA expression. Thus, there seems to be no close correlation between the putative sites of production and actual tissue content, suggesting that activin A is subject to a dynamic pattern of secretion and storage. Further complicating the issue is the fact that follistatin is likewise widely distributed. At least some of the activin A in some of the tissues may be assumed to be already bound to follistatin, either in solution or bound to cell-surface proteoglycans (36). When all tissues are considered, most tissues show a similar ratio of activin A to follistatin, with the notable exception of the bone marrow cells, which show at least a five-fold higher activin A:follistatin ratio than other tissues. This implicates the bone marrow as a major potential source of stored free activin A (i.e., unbound to follistatin) in the normal adult mouse. The inverse implication, that a significant proportion of the activin A present in other

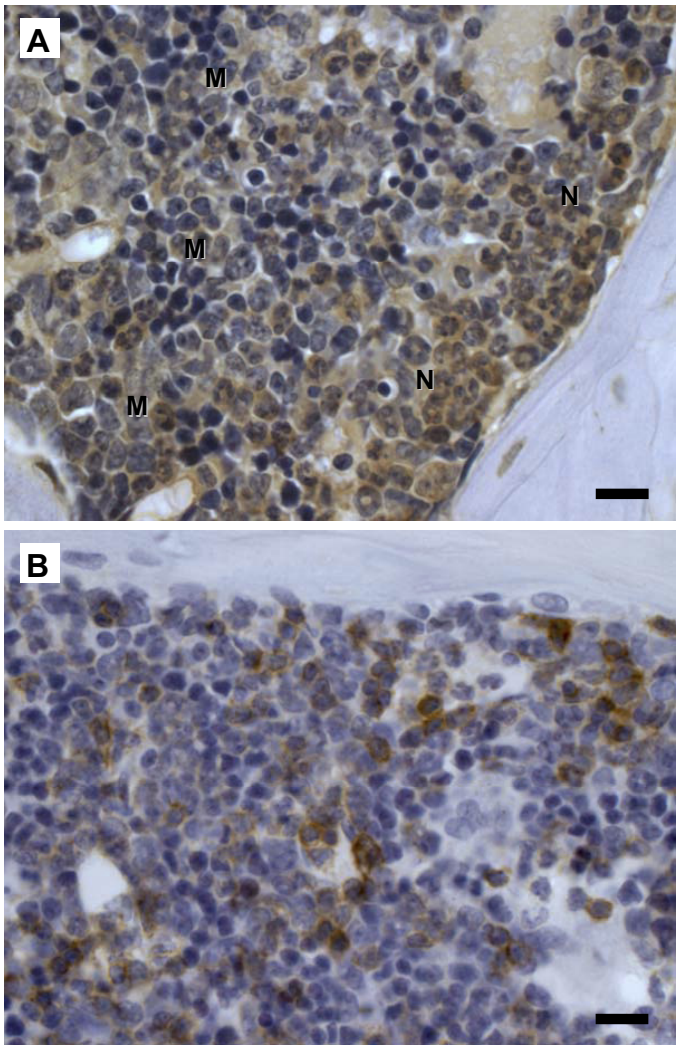

Fig. 8. Immunohistochemical localization of activin A in bone marrow of whole bone sections of control mice and mice injected with LPS 1 h previously. *A*: control mouse bone. *B*: LPS-treated mouse bone. Neutrophil precursor colonies (N), identifiable by the highly polymorphic appearance of the nuclei of their constituent cell types (band neutrophils, segmented neutrophils, and ring-form myelocytes), display activin A labeling. Mononuclear cell colonies (M) are largely unlabeled in bone marrow from control mice. The marrow of LPS-treated mouse bone is characterized by the relative absence of neutrophil precursors, and the increased prominence of activin A-containing cells with mononuclear morphology. Scale bar: ~20 μm.

tissues is actually bound to follistatin, and, hence, may be inactive, also merits consideration.

In contrast to the large response of other rapidly induced inflammatory cytokines, in this case TNF-α and IL-1β, there was no significant increase of activin A mRNA levels in any of the tissues examined within 1 h following LPS challenge. Large variability in mRNA levels is a characteristic of qPCR studies, particularly when dealing with genes that are expressed at low levels, such as activin A, and undoubtedly reflects real differences in mRNA in the source tissues. In the case of the bone marrow and liver, the wide variation and subsequent overlap in mRNA levels in controls and treated samples make it doubtful that these represent meaningful increases or decreases. This was in spite of the fact that serum activin A levels increased at least four-fold at the same time, suggesting that the activin A protein response to LPS stimulation occurs post-mRNA production. This hypothesis was supported by the observation that an inhibitor of mRNA synthesis, actinomycin D, had no significant inhibitory effect on activin A levels in serum after LPS treatment, at a dose that inhibited the increase in both TNF-α and IL-1β.

Post-mRNA regulation could involve new protein translation from the preexisting mRNA in tissues with high levels of endogenous mRNA expression, such as the liver. The other potential source could be the release of prestored activin A protein from cells and tissues. The observation that the protein synthesis inhibitor, cycloheximide, reduced activin A levels following LPS treatment by about 50%, suggest that both mechanisms may be involved. Among the tissues examined, only bone marrow cells, which had the highest activin A levels under normal conditions, had reduced activin A protein 1 h after LPS injection. The only other site that showed a significant change in activin A protein in the first hour after LPS treatment was the lung, with a five-fold increase. These observations suggest that bone marrow could be a major source of released prestored activin A in the serum and in the lungs during acute inflammation induced by LPS, although less significant release of activin A from other tissues as well cannot be excluded.

The failure of activin A gene expression to be immediately up-regulated by inflammatory stimuli has been observed before, for example, in endothelial cells and testicular Sertoli cells (26, 40). This is probably related to the fact that, unlike other rapid response inflammatory cytokines, such as TNF-α and IL-1β, the activin A promoter lacks a response element for the crucial proinflammatory transcription factor, NF-κB, and is more responsive to stress-related signaling pathways, activated via the MAP kinases, with potentially greater latency (10). On the other hand, LPS was able to induce activin A mRNA expression in the liver within 1 h in the presence of cycloheximide, a treatment that also enhanced TNF-α and follistatin expression in response to LPS, implying that protein synthesis-dependent inhibitory regulation may also be involved. Furthermore, the importance of inhibitory mechanisms in control of activin A gene expression is also indicated by the observation that activin A mRNA in the liver actually decreased at 4–5 h after LPS treatment.

The regulation of follistatin was addressed only indirectly in the present study. It has already been established that follistatin is stimulated by LPS and inflammatory cytokines, including TNF-α, IL-1β, and activin A itself, over a much longer time frame than is activin A (13, 15, 29, 40). Accordingly, a significant increase in follistatin mRNA in the liver was first observed at ~5 h in the present study, at the same time as serum follistatin levels increased. However, the data were equivocal, given that neither cycloheximide nor actinomycin D had any inhibitory effect on follistatin levels in the serum following LPS, suggesting that stored protein, rather than new protein synthesis, was largely responsible.

Although actinomycin D alone had no affect on the acute release of activin A or follistatin, it did cause a prolonged release of both activin A and TNF-α from 3 h after LPS until the end of the experiment. This may be attributed to the fact that, in addition to being an mRNA transcription inhibitor, actinomycin D is also able to stabilize preexisting, long-lived

mRNA species, such as activin A mRNA, thereby enhancing protein production from these mRNA (5, 32).

Previous studies have reported that activin A is produced by a number of different bone marrow cells, even under normal conditions, including osteoclasts (11), chondrocytes (44), "monocytoid" cells (39), and stromal or "fibroblastoid" cells (35, 43). Furthermore, blood-derived neutrophils are a source of activin A in humans (4). On the basis of the immunostaining in the present study, it appears that about half of the cells in murine bone marrow express activin A under normal conditions and that labeling is particularly evident in the various neurophil precursors, megakaryocytes, osteoclasts, and the endothelial/fibroblastic cells that line the bone, vasculature, and cell colonies. One hour after an LPS challenge, activin A-containing cells and neutrophil precursors in the bone marrow were greatly reduced in number, and activin A-containing neutrophils appeared throughout the lung. Preferential translocation of neutrophils from the bone marrow to the lungs is a characteristic feature of LPS-induced inflammation and is responsible for lung damage in such models (1, 3, 31, 33). Neutrophils previously have been implicated as a source of activin A in lung inflammation following allergen challenge in humans (14). At least part of the increase in activin A in the blood and in tissues where neutrophils become resident following LPS, most notably the lungs, may be attributed to activin A from these bone marrow cells, in particular. This observation is consistent with accumulating evidence that activin A plays a central role in inflammatory lung damage in various models (2, 9, 18). This does not mean that neutrophils alone are responsible for the increase in activin A following LPS treatment. Release of activin A from other bone marrow cells, such as the stromal cells, also appears to occur. Moreover, it can be expected that cells in other tissues, such as epithelial cells in the lungs and hepatocytes and hepatic stellate cells in the liver (27), for example, may also contribute to this increase.

These data provide novel quantitative information about the sites of production of activin A and follistatin in the mouse. The data indicate that the liver may be the major site of activin A production (i.e., synthesis as opposed to content) under normal conditions in the adult male mouse, in contrast to previous studies in the rat and mouse in which liver expression was reported to be relatively low (19, 34). Another notable observation was that follistatin protein concentrations are normally elevated in tissues of the male reproductive tract, most notably in the vas deferens and prostate. The vas deferens also displayed very high follistatin and activin A mRNA expression, but other organs were significantly lower. This indicates an important role for follistatin in the male tract, which needs to be explored.

Perspectives and Significance

These experiments show that, unlike TNF-α and IL-1β, the increase in activin A within 1 h of LPS injection is not due to an increase in mRNA expression and is most likely due to release of stored activin A and newly synthesized protein from preexisting mRNA. This paper also challenges the widely held notion that mRNA measurements, on their own, are sufficient to understand the complex regulation of cytokines in pathophysiological models. Finally, the discovery of bone marrow-derived cells and the neutrophils, in particular, as the possible source of activin A release during inflammation might lead to the development of new therapeutic targets in acute inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.W., D.J.P., and M.P.H. conception and design of research; H.W., Y.C., W.R.W., D.J.P., and M.P.H. performed experiments; H.W., Y.C., D.J.P., and M.P.H. analyzed data; H.W. and M.P.H. interpreted results of experiments; H.W. prepared figures; H.W. drafted manuscript; H.W., W.R.W., D.J.P., and M.P.H. edited and revised manuscript; H.W., Y.C., W.R.W., D.J.P., and M.P.H. approved final version of manuscript.

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Declaration for Thesis Chapter III

Declaration by candidate

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

Nature of contribution	Extent of contribution (%)
All laboratory work, except as patients' recruitment and blood sample collection. Analysis of data, drafts of the manuscript and preparation of figures for publication.	65

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Michael Wu	Patients' recruitment and blood sample collection	
Yi Chen	Analysis of data	5
Carolyn Allan	Research advice, blood collection of pilot study and final draft of manuscript	
David Phillips	Supervision of research, editing and final draft of manuscript	
Mark Hedger	Supervision of research, editing and final draft of manuscript	

Candidate's Signature	[redacted]	Date 12.02.2013

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 indicat[redacted] / Head of Centre CRD.

Location(s)	[redacted] ment, Monash Institute of Medical Research	
Signature 1	SIGNS ON HIS BEHALF)	Date 13.2.13
Signature 2		13.2.13
Signature 3		12.02.13
Signature 4		13.02.13
Signature 5		13.2.13

Chapter III – Correlation between blood activin levels and clinical parameters of type 2 diabetes

Hypothesis: *Serum activins are elevated in type 2 diabetes, a chronic inflammatory disease, and thereby may act as independent predictors of the disease.*

Background

Type 2 diabetes is a metabolic disorder with chronic inflammation characterised by insulin resistance and hyperglycaemia. Inflammation has been implicated as an important aetiological factor in the development of insulin resistance and onset of T2D. As inflammatory factors, both activins A and B, and their binding protein, follistatin regulate glucose metabolism and inflammation. The activins, and activin B in particular, also have been shown to stimulate insulin gene transcription. In Chapter II, the source and regulation of activin A expression during inflammation was examined in an acute model. This chapter examines the function of activin A, B and follistatin in the chronic inflammatory disease, T2D.

In order to examine this hypothesis, serum activin A and B, and follistatin, were measured in cohorts of patients with insulin resistance or T2D in comparison with normal subjects. Correlations between the serum protein levels and clinical parameters or disease severity were examined.

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Research Article

Correlation between Blood Activin Levels and Clinical Parameters of Type 2 Diabetes

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Aims. Activins A and B, and their binding protein, follistatin, regulate glucose metabolism and inflammation. Consequently, their role in type 2 diabetes (T2D) was examined. *Methods*. Blood was taken from fasted participants (34 males; 58 females; 50–75 years) with diabetes or during an oral glucose tolerance test (OGTT). Clinical parameters were assessed, and blood assayed for activins, follistatin, and C-reactive protein. *Results*. Serum levels of activin A (93.3 ± 27.0 pg/mL, mean ± SD), B (81.8 ± 30.8 pg/mL), or follistatin (6.52 ± 3.15 ng/mL) were not different ($P > 0.05$) between subjects with normal OGTT ($n = 39$), impaired glucose tolerance and/or fasting glucose ($n = 17$), or T2D ($n = 36$). However, activin A and/or activin B were positively correlated with parameters of insulin resistance and T2D, including fasting glucose ($P < 0.001$), fasting insulin ($P = 0.02$), glycated hemoglobin ($P = 0.003$), and homeostasis model assessment of insulin resistance (HOMA-IR; $P < 0.001$). Follistatin was positively correlated with HOMA-IR alone ($P = 0.01$). *Conclusions*. These data indicate that serum measurements of activin A, B, or follistatin cannot discriminate risk for T2D in individual patients, but the activins display a positive relationship with clinical parameters of the disease.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder with chronic inflammation characterised by insulin resistance and hyperglycaemia. Obesity-induced inflammation has been implicated as an important aetiological factor in the development of insulin resistance and onset of T2D [1, 2]. Excessive weight gain leads to increased macrophage infiltration of white adipose tissue, and increased central adiposity is associated with a chronic inflammatory state, induced by proinflammatory mediators, such as tumor necrosis factor (TNF), interleukin 6 (IL-6), and C-reactive protein (CRP) [3, 4]. Inflammatory mediators may also be increased in T2D and insulin resistance, and inhibit insulin signaling through activation of Jun N-terminal kinase (JNK) and nuclear factor (NF)-κB [5, 6], thereby causing insulin resistance and interfering with glucose homeostasis [2, 7, 8]. TNF mRNA is more highly expressed in the fat of obese premenopausal women than that of lean controls, and TNF mRNA expression levels show a positive correlation with hyperinsulinaemia [9]. Furthermore, anti-inflammatory agents such as aspirin or anti-TNF are useful in the control of T2D [10, 11].

Activins A and B are members of the transforming growth factor-β (TGF-β) superfamily and are elevated in the blood and tissues in a broad range of inflammatory diseases [12]. Activin A has long been known to be a critical regulator of inflammation that, like TNF, is upregulated early in inflammation [12, 13]. A potential role for activin B in inflammatory disease has become apparent only more recently. The biological activity of both activins is regulated by a high-affinity activin-binding protein, called follistatin [13].

Activins A and B have been reported to play important roles in glucose metabolism by regulating the differentiation and activity of the insulin-producing β cells and the response of insulin target cells [14, 15]. Administration of follistatin

inhibits the differentiation of pancreas duct epithelial cells into β cells [16]. The activins, activin B in particular, also have been shown to stimulate insulin gene transcription [15]. On the other hand, activin A increases blood glucose level by reducing target tissue sensitivity to insulin [14]. Moreover, activin A affects inflammation in adipose tissue by stimulating macrophages to switch from a proinflammatory phenotype to an anti-inflammatory phenotype [17]. Thus, obesity-induced inflammation may provide a bridge between T2D and activin activity. In addition, activation of inflammation through the toll-like receptor (TLR) 4 pathway can stimulate release of activin A [12]. TLR4 mRNA and protein expression and TLR4 signaling were increased in recently-diagnosed T2D and TLR4 levels were positively correlated with the glucose level and severity of insulin resistance in this population [18].

As a consequence of their multiple roles, the activins could provide an important link between inflammation, glucose metabolism and T2D. Notably, follistatin is correlated with insulin resistance in patients with polycystic ovary syndrome [19]. In order to identify potential roles for the activins and follistatin in onset and established T2D, the following study examined these proteins, as well as several crucial functional parameters, in a small population of subjects with normal glucose tolerance, impaired fasting glucose (IFG), and/or impaired glucose tolerance (IGT), and in patients with T2D. These data indicated a positive relationship between the activins and clinical parameters of T2D, indicating that more detailed investigation of the role of the activins in T2D could be of considerable value.

2. Materials and Methods

These studies were approved by the Human Research and Ethics Committee, Monash Medical Centre, Melbourne and adhered to the principles of the Declaration of Helsinki.

2.1. Pilot Study. In order to investigate whether acute changes of glucose and insulin levels affect the levels of the activins and follistatin in serum, five healthy men aged 25–50 years were given a standard oral glucose tolerance test (OGTT) with 75 g glucose in 300 mL water. Venous blood samples were collected at 0 min, 60 min and 120 min [20].

2.2. Main Study. Participants were recruited through advertisement from the community and ambulatory care clinics. Inclusion criteria for participants were as follows: between 50 to 75 years of age, postmenopausal for at least six months, nonsmokers for at least one year, and no changes to prescribed medications in the previous three months. Participants were excluded if they had type 1 diabetes mellitus, previous arterial surgery, known renal impairment (eGFR < 60 mL/min), unstable angina, class 3 or 4 New York Heart Association congestive heart failure, severe peripheral vascular disease (rest pain or active ulceration), presence of inflammatory diseases (e.g., rheumatoid arthritis), or regular usage of nonsteroidal anti-inflammatories or prednisolone. After informed consent was obtained in writing from each participant, all participants underwent a baseline assessment that included a medical history, blood pressure measurement, calculation of body mass index (BMI) and waist: hip ratio (WHR). BMI was calculated as weight in kilograms divided by the square of height in metres. All subjects had fasting serum and plasma (in EDTA) samples and urine samples collected on the morning of study. Subjects were advised to avoid caffeine-containing drinks during a 12 hour overnight fast prior to the study. An OGTT was administered to the participants who had no known history of T2D [20]. Venous blood samples were collected at 0 min, 60 min and 120 min [20]. Participants underwent only fasting blood collection if they had a known history of T2D.

2.3. Lifestyle Factors. Lifestyle factors were annotated to include any history of smoking, the level of exercise (score $1 \leqslant 1$ hour/week, 2 = 1–3 hours/week, $3 \geqslant 3$ hours/week), and alcohol intake (score 1 = never, 2 = 1 drink/week, 3 = 1-2 drinks/week, 4 = 3-4 drinks/week, 5 = 5-6 drinks/week, 6 = daily drinking).

2.4. Biochemical and Immunoassay Measurements. Activin A was measured by a two-site ELISA (Oxford Bio-innovations, Cherwell, Oxfordshire, UK), using human recombinant activin A as standard [21]. This assay measures both free and follistatin-bound activin A dimers and has no significant cross-reaction with other isoforms of activin, such as activin B. The mean assay sensitivity was 12 pg/mL. The mean intra-assay and interassay coefficients of variation (CVs) were 9.5% and 6.3%, respectively.

Activin B was measured by a two-site ELISA (Oxford Bio-innovations, Cherwell, Oxfordshire, UK), using human recombinant activin B as standard [22]. This assay measures both free and follistatin-bound activin B dimers and has no significant cross-reaction with other isoforms of activin. The mean assay sensitivity was 12 pg/mL. The mean intra-assay and interassay coefficients of variation (CVs) were 6.7% and 4.1%, respectively.

Follistatin was measured by radioimmunoassay (RIA) [23]. This assay uses human recombinant follistatin (supplied by the National Hormone and Peptide Program, NHPP) as standard and tracer. The mean assay sensitivity was 0.97 ng/mL. Mean intra- and interassay CVs for follistatin were 9.4% and 8.4%, respectively.

All other clinical measurements were performed by the clinical laboratory of the Monash Medical Centre Pathology Department). Plasma glucose was measured with the glucose oxidase method. Insulin was measured using the Access/DXI Ultrasensitive one-step immunoenzymatic assay (Beckman Coulter Diagnostics Australia). Glycated haemoglobin (HbA1c) was measured via a cation exchange column, based on high performance liquid chromatography (TOSO Corporation). Total cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides were measured using a commercial enzymatic assay (Beckman Coulter Diagnostics Australia). Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation [24]. Serum creatinine was measured with an automated colorimetric method (Beckman Coulter SYNCHRON LX20PRO, Sydney,

Australia) using a modified kinetic Jaffe reaction. Renal function was expressed as estimated glomerular filtration rate (eGFR) based on the Modification of Diet in Renal Disease study [25]. High sensitivity C-reactive protein (hsCRP) was measured using a Near Infrared Particle Immunoassay (Beckman Coulter Diagnostics Australia). Urinary assays were corrected for individual urinary creatinine levels.

Homeostasis model assessment of insulin resistance (HOMA-IR) was estimated with the following formula: insulin resistance = fasting plasma insulin (in microunits per ml) × fasting plasma glucose (FPG, in millimoles per litre)/22.5. Patients were assessed as normal if HOMA-IR was <2.5, and insulin-resistant if HOMA-IR was ≥2.5) [26]. Area under the curve for OGTT glucose levels (AUCg) was calculated using trapezoidal integration [27].

2.5. Statistical Analyses. Continuous data are reported as mean ± standard deviation and categorical data as percentages. Mean comparisons were made by independent samples *t*-test and analysis of variance (ANOVA), as appropriate. Pearson correlations were used to investigate the associations between the activins, follistatin, and confounding variables, including age, history of smoking, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), centrally-derived systolic blood pressure (cSBP), use of medications, and diabetes clinical parameters. Multivariate models were subsequently generated by multiple linear regression analysis to identify independent relationships between the activins, follistatin and the clinical parameters. All statistical tests were two-sided and *P* values of less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 19 (SPSS Inc., Chicago, US) or GraphPad Prism 5 (GraphPad, San Diego, US).

3. Results

3.1. Subject Characteristics. Thirty-nine subjects had a normal OGTT, 17 subjects had impaired fasting glucose/impaired glucose tolerance (IFG/IGT), and 36 subjects had T2D according to the American Diabetes Association (ADA) and WHO criteria (Table 1) [28, 29]. Amongst the 36 subjects with T2D, 24 were on oral hypoglycaemic medication, either metformin or sulphonylureas, or both. On average, subjects in the T2D group were older and had higher WHR, HbA1c, AUCg, fasting glucose, fasting insulin, triglycerides, HOMA-IR and lower total cholesterol, HDL and LDL levels than those in the normal group, and were more likely to be using anti-hypertensive medication. There were no differences between the groups in level of exercise or current alcohol consumption, history of smoking, BMI, Crcl (creatinine clearance), and HsCRP ($P > 0.05$, not shown).

3.2. Responses to Acute Changes in Glucose and Insulin Levels in Normal Subjects. Acute changes of glucose and insulin levels following an OGTT did not affect the concentrations of activin A, activin B or follistatin in the circulation of normal participants in the subsequent 2 h period (Figure 1).

3.3. Correlations of the Activins and Follistatin with General Subject Characteristics. In the total study population ($n = 92$), serum levels (mean ± SD) of activin A, activin B, and follistatin were 93.3 ± 27.0 pg/mL, 81.8 ± 30.8 pg/mL, and 6.52 ± 3.15 ng/mL, respectively. There were no significant differences in serum activin A, activin B, follistatin, and HsCRP between the normal, impaired glucose tolerance (IFG/IGT), and T2D groups in the subject population (Table 1), although there was clear evidence of a trend towards an increase in both activins and follistatin in the T2D group compared with the other two groups.

Serum levels of activin A, but not activin B or follistatin, were positively correlated with age in normal subjects and the IFG/IGT group, but not in the T2D group (Tables 2–4). Activin A and follistatin, but not activin B, were also significantly correlated with BMI and past history of smoking in T2D subjects.

Activin A and B and follistatin showed no association with gender, WHR, alcohol consumption, level of exercise, serum cholesterol, HDL, LDL, triglycerides, or antiglycaemic medication used in any group ($P > 0.05$, data not shown), with the following exceptions: activin B showed a weak negative correlation with HDL in total subjects ($r = -0.224$, $P < 0.05$), and follistatin showed a negative correlation with HDL in normal subjects only ($r = -0.364$, $P < 0.05$) and a positive correlation with triglycerides in the T2D group only ($r = 0.410$, $P < 0.05$).

Activin A, but not activin B or follistatin, was also significantly elevated in subjects with a history of hypertension ($P < 0.001$). In a parallel study by some of the authors (M. Wu et al., submitted manuscript), activin A, but not activin B or follistatin, was found to be positively correlated with the following blood pressure variables: cSBP, PP, and MAP. There was a positive relationship between activin A levels and the use of ACE inhibitors ($r = 0.347$, $P < 0.001$), beta blockers ($r = 0.355$, $P < 0.001$) and/or diuretics ($r = 0.344$, $P < 0.001$), but not with the use of statins, calcium-channel blockers or aspirin ($P > 0.05$). Activin B was significantly elevated in subjects using diuretics only ($P < 0.01$), and follistatin was not affected by any of these medications.

3.4. Correlations of the Activins and Follistatin with Functional Parameters of T2D and Insulin Resistance. There was no relationship between activin A and B in normal subjects or in the IFG/IGT group, but activin A levels overall were positively correlated with activin B as a result of a very strong relationship within the T2D group (Figure 2 and Table 2). Neither activin A nor activin B showed a correlation with follistatin in any group (Tables 2 and 3).

Activin A and B were correlated with most functional parameters of T2D and insulin resistance (fasting glucose, fasting insulin, HbA1c, and HOMA-IR, although not AUCg) among total participants and in the T2D group, specifically, with the single exception that activin B was not significantly correlated with fasting insulin in the T2D group (Tables 2–4). Activin A and B were also correlated with fasting insulin and HOMA-IR in the IFG/IGT group. After adjusting for covariants/confounding factors (including age, BMI, history of smoking, history of hypertension and medication use,

Table 1: Subject characteristics.

	Normal ($n = 39$)	IFG/IGT ($n = 17$)	T2D ($n = 36$)	P value
Age (years)	57.0 ± 5.8	58.0 ± 7.1	62.6 ± 8.4	<0.05
Male gender (%)	38	35	64	—
WHR	0.88 ± 0.08	0.88 ± 0.07	0.95 ± 0.07	<0.001
BMI (kg/m^2)	27.4 ± 5.5	28.3 ± 3.1	30.0 ± 4.8	NS
History of smoking	13	8	16	—
Fasting glucose (mmol/L)	5.2 ± 0.5	5.7 ± 0.5	7.8 ± 2.3	<0.001
HbA1c (%)	5.6 ± 0.3	5.9 ± 0.3	7.3 ± 0.9	<0.001
Fasting insulin (mU/L)	5.5 ± 3.2	6.2 ± 2.5	8.6 ± 5.4	<0.001
HOMA-IR	1.3 ± 0.77	1.6 ± 0.66	3.1 ± 2.4	<0.001
AUCg[†]	872.0 ± 132.3	1075.5 ± 147.8	1399.5 ± 205.6	<0.001
History of hypertension	7	6	28	—
Total Chol (mmol/L)	5.2 ± 0.8	5.2 ± 1.0	4.4 ± 1.0	<0.001
LDL (mmol/L)	3.3 ± 0.77	3.1 ± 0.9	2.6 ± 0.9	<0.05
HDL (mmol/L)	1.5 ± 0.4	1.4 ± 0.4	1.1 ± 0.2	<0.001
TG (mmol/L)	1.0 ± 0.5	1.6 ± 0.7	1.5 ± 0.9	<0.05
Crcl (mL/min)	97.0 ± 33.4	99.2 ± 30.0	94.8 ± 40.6	NS
HsCRP (ng/mL)	3.0 ± 4.5	2.1 ± 3.8	2.7 ± 4.8	NS
Antihyperglycaemic use	0	0	24	—
Antihypertensive use	5	3	14	—
Activin A (pg/mL)	90.1 ± 24.8	89.6 ± 20.3	98.4 ± 31.6	NS
Activin B (pg/mL)	79.5 ± 29.5	76.1 ± 27.5	87.3 ± 33.8	NS
Follistatin (ng/mL)	6.2 ± 2.4	7.0 ± 2.5	6.7 ± 4.1	NS

WHR: waist to hip ratio; BMI: body mass index; HbA1c: glycated hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance; AUCg: area under the curve for OGTT glucose levels; SBP: systolic blood pressure; DBP: diastolic blood pressure; Chol: cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; TG: triglycerides; Crcl: creatinine clearance; HsCRP: human sensitive C-reactive protein. [†]Note that only four patients in the T2D patients group were given an OGTT, as most of these patients had a prior diagnosis of T2D. Data are mean ± SD. One way ANOVA was used for analysis. NS: $P > 0.05$.

blood pressure parameters), activin A lost its correlation with HbA1c but retained a positive association with fasting glucose, fasting insulin and HOMA-IR, due almost entirely to very strong correlations in the T2D group (Figure 2). After adjustment, activin B lost its correlation with fasting insulin, but retained a positive association with fasting glucose, HbA1c and HOMA-IR. Follistatin was positively correlated with HOMA-IR and AUCg among total participants only, and this was not altered after multivariate analysis.

Follistatin was positively correlated with HsCRP levels in all the groups of subjects, but neither activin A nor activin B showed significant correlation with HsCRP (Tables 2–4). Neither activin A nor activin B was significantly correlated with the kidney function parameters, Crcl and eGFR (data not shown), whereas follistatin was positive correlated with Crcl (Tables 2–4).

4. Discussion

This study examined the levels of activin A, activin B, and follistatin in the blood of normal subjects, and in patients with IFG/IGT or T2D. Despite circulating concentrations of these proteins not being different between the three groups, significant correlations between the levels of these proteins and several functional parameter of T2D and insulin resistance were noted, such as fasting glucose, fasting insulin, HbA1c, and HOMA-IR. The data suggest that a much larger sample population should produce significant differences between the mean activin and follistatin levels in normal and T2D patient groups. A number of other correlations were also apparent. This differs from a previous study in patients with acute myocardial infarction whereby activin A was elevated in the group with higher blood glucose levels, but is consistent with a study showing that blood activin A levels were not altered in T2D subjects compared with control subjects [30, 31]. The difference might be due to the different inflammatory conditions involved, since acute myocardial infarction causes acute inflammation and oxidative stress, whereas type 2 diabetes is a chronic inflammatory disorder [2, 30]. Although the serum levels of CRP, commonly used as a marker of inflammation, were not significantly increased in the T2D groups, it should be noted that an increase in CRP is not a universal feature of T2D [32, 33]. This suggests that inflammation may not be the major factor leading to the T2D observed, at least in this particular set of subjects. In the present study, however, a significant relationship between hsCRP and follistatin, but not the activins, was observed.

The correlations are consistent with a previous study which showed activin B subunit mRNA expression in adipose tissue was positively correlated with adipose and serum fasting insulin level [34]. These observations of a positive correlation between activin A or B or follistatin and T2D

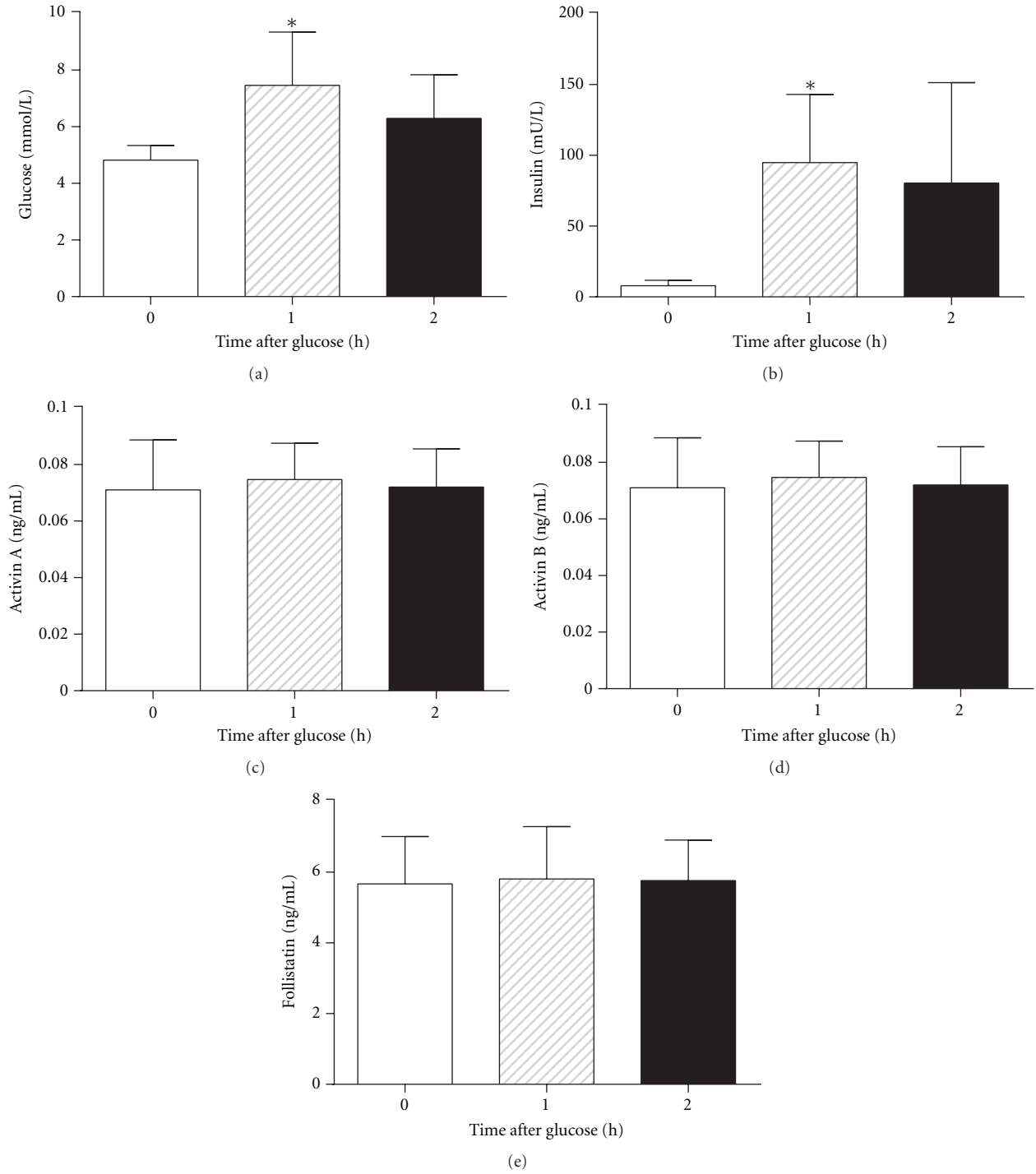

FIGURE 1: Blood levels of (a) glucose, (b) insulin, (c) activin A, (d) activin B, and (e) follistatin during an OGTT of normal participants (pilot study). All data are mean ± SD, $n = 5$ subjects. $^*P < 0.05$ compared with 0 h value.

and insulin resistance parameters indicates that the activins might be a clinical indicator for the severity of T2D, which may not only indicate the glucose control condition but also reveal the severity of insulin resistance, and that larger clinical trials of this relationship are definitely warranted.

The results also show that activin A is positively correlated with age in all the participants except the T2D group, which is consistent with our data showing that in a healthy population, and activin A is elevated with increasing age [35]. While activin B has no correlation with age [22]. This is also the first time activin B and follistatin have been examined for a relationship with past smoking. Our study shows that none of anti-diabetic medications affect the level of activins or follistatin, although previous data showed that metformin

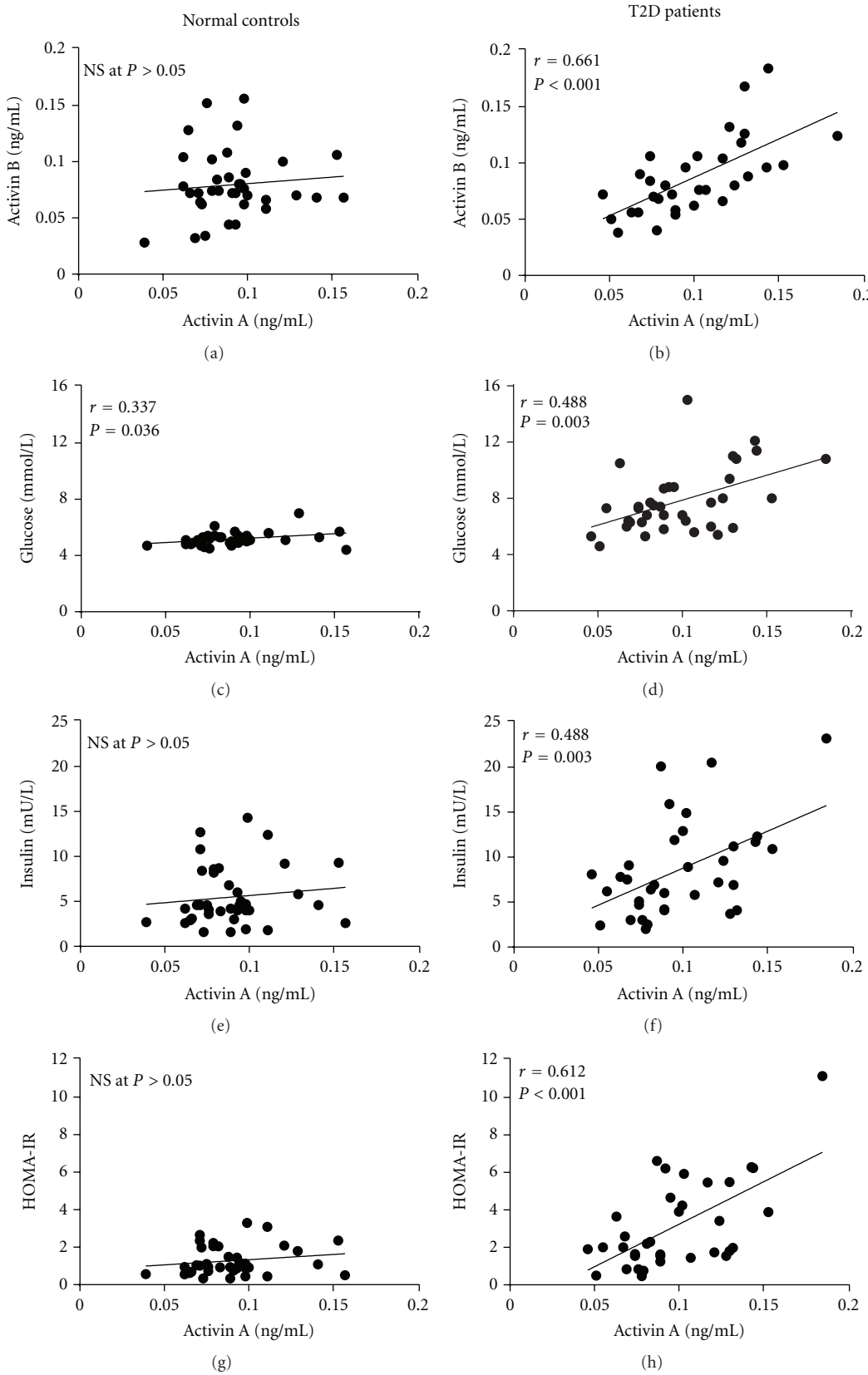

Figure 2: Individual subject data for the correlations of blood levels of activin A with activin B (a, b), glucose (c, d), insulin (e, f), and HOMA-IR (g, h) in normal controls and T2D patients. Note that this figure presents some data from Table 2 in graphical form.

Table 2: Associations between circulating levels of activin A and demographic and biochemical indices within subject groups.

	Total subjects	Normal	IFG/IGT	T2D
Age	0.376***	0.329*	0.550*	0.320
BMI	0.346***	0.227	0.362	0.434**
Smoking	0.205*	0.183	−0.250	0.352*
Fasting glucose	0.388***,†	0.337*	−0.044	0.488**,†
HbA1c	0.386**	0.086	0.084	0.588**
Fasting insulin	0.399***,†	0.123	0.589*	0.488**,†
HOMA-IR	0.485***,†	0.177	0.547*	0.612***,†
AUCg	−0.070	−0.009	0.059	−0.195
Crcl	0.045	−0.076	−0.030	0.168
HsCRP	0.110	−0.004	0.380	0.138
Activin B	0.430***	0.099	0.428	0.661***
Follistatin	0.196	−0.028	0.280	0.291

BMI: body mass index; HbA1c: glycated hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance; AUCg: area under the curve for OGTT glucose levels; Crcl: creatinine clearance; HsCRP: human sensitive C-reactive protein. Data presented as univariate Pearson correlation coefficients (R) value: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Correlations with no superscript are not significant ($P > 0.05$). †After adjustment for confounding factors (including age, BMI, history of smoking, history of hypertension and medication use, blood pressure parameters) by multivariate analysis, activin A retained a positive association with fasting glucose, fasting insulin, and HOMA-IR but lost its correlation with HbA1c.

Table 3: Associations between circulating levels of activin B and demographic and biochemical indices within subject groups.

	Total subjects	Normal	IFG/IGT	T2D
Age	0.100	−0.206	0.267	0.159
BMI	0.170	0.092	0.321	0.201
Smoking	0.160	0.029	0.356	0.200
Fasting glucose	0.298**,†	0.072	0.080	0.406*
HbA1c	0.331**,†	−0.193	0.292	0.529**
Fasting insulin	0.254*	0.051	0.510*	0.293
HOMA-IR	0.332**,†	0.056	0.514*	0.426*,†
AUCg	−0.100	0.066	−0.155	−0.274
Crcl	0.009	0.098	−0.121	0.004
HsCRP	0.120	0.021	−0.243	−0.119
Follistatin	0.026	0.008	−0.010	0.033

BMI: body mass index; HbA1c: glycated hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance; AUCg: area under the curve for OGTT glucose levels; Crcl: creatinine clearance; HsCRP: human sensitive C-reactive protein. Data presented as univariate Pearson correlation coefficients (R) value: $^{*}P < 0.05$, $^{**}P < 0.01$. Correlations with no superscript are not significant ($P > 0.05$). †After adjustment for confounding factors (including age, BMI, history of smoking, history of hypertension and medication use, blood pressure parameters) by multivariate analysis, activin B retained a positive association among total subjects with fasting glucose, HbA1c, and HOMA-IR but lost its correlation with fasting insulin. In the T2D group, only HOMA-IR retained a positive correlation with activin A after adjustment.

was able to reduce activin A release from monocytes *in vitro* [31]. Aspirin, likewise, was not significantly correlated with any of the glucose parameters, which is different from data in rats showing that aspirin is able to improve the clinical glucose and insulin resistance parameters in T2D [10].

Table 4: Associations between circulating levels of follistatin and demographic and biochemical indices within subject groups.

	Total subjects	Normal	IFG/IGT	T2D
Age	0.028	−0.041	0.399	−0.054
BMI	0.200	0.004	0.140	0.373*
Smoking	0.304**	0.162	0.226	0.413*
Fasting glucose	0.130	−0.221	0.172	0.175
HbA1c	0.100	−0.061	0.274	0.073
Fasting insulin	0.180	0.082	0.304	0.187
HOMA-IR	0.268*	0.041	0.351	0.323
AUCg	0.271*	0.071	0.411	−0.042
Crcl	0.210	−0.011	0.095	0.371*
HsCRP	0.585***	0.394*	0.642**	0.740***

BMI: body mass index; HbA1c: glycated hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance; AUCg: area under the curve for OGTT glucose levels; Crcl: creatinine clearance; HsCRP: human sensitive C-reactive protein. Data presented as univariate Pearson correlation coefficients (R) value: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Correlations with no superscript are not significant ($P > 0.05$). Follistatin was positively correlated with HOMA-IR and AUCg among total participants only, and this was not altered after multivariate analysis for confounding factors (including age, BMI, history of smoking, history of hypertension and medication use, blood pressure parameters).

Finally, this study showed that activins A or B or follistatin were significantly correlated with cardiovascular disease risk factors, such as obesity (BMI), smoking, and lipid profiles (HDL and triglyceride).

The fact that serum levels of activins or follistatin are not clearly predictive of T2D or insulin resistance in individual patients could be due to the complicated roles of activin A and B in the regulation of glucose and insulin metabolism. As previously described, activin A and B both have the ability to improve insulin resistance and help release of insulin to control the blood glucose, but they also promote inflammation in the insulin-responsive tissues and contribute to insulin resistance [14]. This study also suggests that activin A and activin B were not changed with acute changes of glucose and insulin. This differs from a previous study in patients with acute myocardial infarction whereby activin A was elevated in the group with higher blood glucose levels, but is consistent with a study showing that blood activin A levels were not altered in T2D subjects compared with control subjects [30, 31]. This can be explained by the fact that activins are inflammatory factors which would be elevated in an inflammatory response, whereas acute changes of glucose or insulin levels will not cause inflammation in normal subjects. This is also the first clinical study to measure activin B in glucose metabolism, whereas previous studies have examined activin A in glucose metabolism, and follistatin in insulin resistance [19, 30, 31].

The correlations between the activins, follistatin, T2D, and insulin sensitivity indicate a complex set of mechanisms is involved. Activin A and B promote β-cell differentiation, insulin gene expression, and insulin secretion from β cells [14, 15]. Conversely, in the insulin-responsive tissues, liver, skeletal muscles, and adipose tissue, activin shrinks the size of the organ thereby reducing insulin sensitivity [14]. The

effect of activin A on glucose metabolism might also be due to one of the nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR)γ, which improves insulin sensitivity by increasing peripheral glucose disposal and decreasing hepatic glucose production [36]. It has been shown that activin A is able to inhibit the expression of PPARγ [37]. Further, activins' contribution to insulin resistance could also be the consequence of activation of one of the adipokines, plasminogen activator inhibitor 1 (PAI-1). In obese subjects, increased expression of PAI-1 in adipose tissue is associated with insulin resistance [38]. After binding to activin receptors, activins activate Smad intracellular signaling molecules, and Smad 3 and 4 are able to enhance the expression of PAI-1 in adipose tissue, one of the insulin target tissues [39]. As a result, activin A and B might increase insulin resistance by stimulating expression of PAI-1. A further mechanistic consideration is that free fatty acid (FFA) levels, which are increased in T2D and central obesity, might play an important role in driving activin and FS changes in T2D. FFA is able to stimulate Toll-like receptor 4 (TLR4), a key signaling molecule in the innate immunity pathway, which is also known to stimulate the release of activin A [1, 12].

In conclusion, this study indicates that measurements of activin A, B, or follistatin cannot discriminate risk for T2D in individual patients, but nonetheless suggests that the activins and follistatin might have important roles in insulin resistance and the onset and development of T2D. Clinically, activin A, B, or follistatin may be useful as indicators of the severity of T2D and insulin resistance. Furthermore, the tissue specific manner of activins's function of modulating insulin sensitivity and insulin secretion may help identify the target tissue for prevention and management of T2D and insulin resistance. Therefore, further studies to investigate the role of the activins, as well as its binding protein follistatin, in this disease may be valuable for the development of future diagnostics or therapeutics.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Declaration for Thesis Chapter IV

Declaration by candidate

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

Nature of contribution	Extent of contribution (%)
All laboratory work, except some activin and follistatin protein assays. Design of experiments and research planning, analysis of data, drafts of the manuscript and preparation of figures for publication.	70

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Yi Chen	Assistance with blood collection, bone marrow collection and cell culture	10
Wendy Winnall	Research advice and assistance with cell culture	
David Phillips	Supervision of research, editing and final draft of manuscript	
Mark Hedger	Supervision of research, editing and final draft of manuscript	

Candidate's Signature	[redacted]	**Date** 12.02.2013

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)	**Centre for Reproduction and Development, Monash Institute of Medical Research**	
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Signature 2		14.2.13
Signature 3		13.02.13
Signature 4		13.2.13

Chapter IV – Regulation of activin A release from murine bone marrow-derived neutrophil precursors by tumour necrosis factor-α and insulin

Hypothesis: *Neutrophils are a source of activin A, which is regulated by inflammatory stimuli. Glucose and/or insulin regulate activin A production from neutrophils.*

Background

In Chapter II, neutrophils were implicated as a major source of activin A during inflammation in the mouse. In the human, studies in our laboratory have shown that neutrophils contain activin A that can be released in response to TNF-α, although these cells do not respond directly to LPS. Studies of inflammatory disease *in vivo*, however, generally use the mouse, so it was essential to know if murine neutrophils have similar properties.

The response of murine bone marrow-derived neutrophil precurosrs (BMNPs) to TNF-α and LPS was examined in culture. As demonstrated in Chapter III, activin A is implicated in T2D, a disease characterised by insulin resistance, hyperglycaemia and chronic elevation of pro-inflammatory cytokines, including TNF-α. The ability of insulin and glucose to modulate an activin A response in these cells, which are de-regulated in T2D, was also investigated.

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Regulation of activin A release from murine bone marrow-derived neutrophil precursors by tumour necrosis factor-α and insulin

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Lipopolysaccharide

ABSTRACT

Activin A, a transforming growth factor-β family cytokine, plays a crucial role in regulating the onset and severity of many inflammatory conditions, such as acute lipopolysaccharide (LPS)-induced inflammation. Activin A is also implicated in type 2 diabetes (T2D), a disease characterised by insulin resistance, hyperglycaemia and chronic elevation of pro-inflammatory cytokines, including tumour necrosis factor (TNF-α). In the human, neutrophils contain activin A that can be released in response to TNF-α. Studies of inflammatory disease *in vivo*, however, generally use the mouse, so it is essential to know if murine neutrophils have similar properties. Regulation of activin A was investigated in bone marrow-derived neutrophil precursors (BMNPs) from 8 to 10 weeks old C57BL6/J male mice. The BMNPs contained 7-fold higher concentrations of activin A than bone marrow mononuclear cells. Release of activin A from isolated BMNPs was stimulated by TNF-α, but this was not due to increased activin A production. In contrast to TNF-α, LPS had no effect on isolated BMNPs, but stimulated activin A release and production in total bone marrow cell cultures. Moreover, activin A release in response to LPS, was not prevented in TNF-α null mice. Increased glucose and insulin had no effect on base-line activin A secretion by BMNPs in culture, but pre-treatment with insulin blocked the TNF-α induced release of activin A. These results indicate that murine neutrophils are a source of stored activin A, the release of which can be directly stimulated by TNF-α, although TNF-α is not the only stimulator of activin A release during inflammation. Furthermore, regulation of neutrophil activin A release by insulin may also play a role in the inflammation associated with T2D.

1. Introduction

Activin A is a disulphide-linked homodimeric protein, and a member of the transforming growth factor-β (TGF-β) superfamily of cytokines [1]. It is widely produced and distributed in the body and has been shown to play critical roles in embryo development, liver function, reproduction and immunoregulation. Activin A levels are increased in a wide range of inflammatory diseases [1]. During acute inflammation caused by administration of lipopolysaccharide (LPS), there is a biphasic release peak of activin A into the blood and the early release of activin A coincides with the release of the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α) [2]. In a mouse model of LPS-induced endotoxaemia, treatment with the activin A binding protein, follistatin, modulated the expression of pro-inflammatory cytokines such as TNF-α, and reduced the mortality rate [3]. These data suggest that activin A plays a crucial role in regulating the onset and severity of inflammatory diseases.

The bone marrow is a major source of activin A during inflammation in mice, and both murine and human neutrophils have been shown to contain activin A (AJP ms) [4,5]. Preferential translocation of neutrophils from the bone marrow to the lungs is a characteristic feature of LPS-induced inflammation, which is responsible for lung damage [6–9], and is matched by a significant increase in activin A in the lungs in such models [5] (AJP ms). Neutrophils have also been implicated as a source of activin A during lung inflammation following allergen challenge in humans [10]. In cultured human neutrophils, TNF-α is able to stimulate activin A release [4], whereas blocking activin A with follistatin in the mouse model of endotoxaemia reduced serum TNF-α levels [3]. These studies suggest that there is an interactive relationship between TNF-α and activin A, involving the neutrophils, during inflammation.

An interactive relationship between TNF-α, activin A and neutrophil function also has been recognised in type 2 diabetes (T2D). This is a disease characterised by raised insulin, hyperglycaemia and elevated expression of pro-inflammatory cytokines,

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including TNF-α [11]. A study in patients with acute myocardial infarction showed that higher activin A levels were associated with higher blood sugar levels [12], and, in a cohort of patients with T2D, we have observed that levels of activin A in the blood are positively correlated with the severity of the disease (Wu H. et al., manuscript submitted for publication). Furthermore, studies have shown that insulin inhibits the release of TNF-α and other pro-inflammatory cytokines in response to LPS in mice [13]. On the other hand, insulin was shown to reduce LPS-induced oxidative responses and inflammatory stress, but had no depressive effects on LPS-induced TNF-α and other pro-inflammatory cytokines in human studies [14]. Furthermore, it has been demonstrated that insulin treatment is able to attenuate systemic inflammatory responses and modulate monocyte/macrophage and neutrophil function [15,16]. Notably, insulin can bind to receptors on the surface of human neutrophils and attenuate functions such as chemotaxis and phagocytosis [16]. These studies suggest that elevated insulin and glucose during T2D could play a role in regulating neutrophil function and their production of activin A in this metabolic disease, which also appears to be chronic inflammatory disease.

The mouse is widely used for studies of inflammatory diseases. In this study, we investigated whether murine neutrophils are a source of activin A that may be regulated during inflammatory disease, by studying the response of murine bone marrow-derived neutrophil precursors (BMNPs) to TNF-α and LPS. Moreover, we examined whether TNF-α is essential for inducing activin A release during inflammation *in vivo* in LPS-treated mice lacking TNF-α. The ability of insulin and glucose, which are dysregulated in T2D, to modulate activin A responses in these cells was also investigated.

2. Materials and methods

2.1. Animals

Adult male mice (8–10 weeks old; 18–22 g) of the C57BL6/J strain were employed for the isolation of bone marrow-derived cells. The TNF-α null (TNF-α −/−) mice, which were generated by direct gene targeting of C57BL6 embryonic stem cells, were a kind gift from Dr. Peter Tipping (Department of Medicine, Monash University) [17]. Procedures involving animals were conducted in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care of Scientific Purposes (1997) and were approved by the Monash Medical Centre Animal Ethics committee.

2.2. Reagents

RPMI-1640, sodium bicarbonate, penicillin/streptomycin antibiotics and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA, USA). Mouse recombinant TNF-α was obtained from R&D systems (Minneapolis, MN, USA). LPS (from *E. coli*, serotype 0111:B4), glucose and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St Louis, MO, USA). Insulin was obtained from Eli Lilly Australia Pty. Ltd. (West Ryde, NSW, Australia).

2.3. Extraction of bone marrow cells, neutrophils and mononuclear cells

Bone marrow-derived cells were collected by syringe flushing of the femurs of 8–10 weeks old male mice by injection of phosphate-buffered saline (PBS), and then centrifuged at 340*g* for 5 min at 4 °C. The BMNPs (myelocytes and band neutrophils) were extracted from total bone marrow cells and isolated and purified by magnetic cell sorting using the reagents and protocol of the supplier (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) [18]. Cell pellets were dissolved in phosphate-buffered saline (PBS), pH 7.2, containing 0.5% BSA, 2 mM EDTA (200 μL buffer per 10^8 total cells) and incubated with mouse anti-Gr-1-biotin (50 μL of stock solution per 10^8 total cells) at 4 °C for 15 min. The Gr-1 antibody reacts with Ly-6G, a myeloid differentiation antigen expressed on mature granulocytes in bone marrow and tissues [19]. Thereafter, 150 μL of buffer and 100 μL of stock solution of anti-biotin magnetic microbeads per 10^8 total cells were added for a further 15 min incubation at 4 °C. After incubation, cells were washed with 1 mL buffer and centrifuged at 340*g* for 5 min and the supernatant removed. After resuspension in 500 μL buffer, the cells were applied to a magnetic separation column (MACS MS column, Miltenyi Biotec). The BMNPs were retained by the magnetic separation column and unlabeled mononuclear cells (monocyte and erythroid precursors) passed through the column without adhesion and were collected in 1.5 mL tubes. The column was washed with 500 μL buffer three times and after these washes the column was removed from the magnetic separator and placed in a 1.5 mL tube. Finally, 1 mL of buffer was pipetted onto the column and immediately flushed through to elute the BMNPs. The purity of the neutrophils and mononuclear cell isolates were determined by flow cytometry (Abbott Cell-Dyn 3200 flow cytometer, Abbott Park, IL, USA) and confirmed by manual counting of haematoxylin-stained cytospin preparations [4].

2.4. Cell cultures

Cells were incubated (37 °C, pH 7.4, 5% CO_2 in air) in Falcon 96-well culture plates (Becton–Dickinson Labware, Bedford, MA) at the density of two million cells per well in 0.2 mL of serum-free RPMI-1640 (pH 7.3–7.4) with penicillin (10,000 U/mL), streptomycin (10,000 μg/mL) and 0.2% sodium bicarbonate. Isolated BMNPs (purity > 98%) and bone marrow-derived mononuclear cells (consisting of 50–70% lymphocytes and 10–50% monocytes) were cultured without addition, or in the presence of TNF-α (100 ng/mL) or LPS (100 ng/mL) for 1, 4 and 12 h. The doses of LPS and TNF-α used were established from dose–response studies on human neutrophils [4]. In some experiments, cells were also treated with insulin (0.5 mU/mL) or glucose (25 mM). Insulin dose–response studies established that there was no difference in response between the concentration of 0.5 and 500 mU/mL; consequently, 0.5 mU/mL was chosen as the experimental dose, which is closer to the physical condition of insulin resistance since blood insulin concentrations are usually more than 0.1 mU/mL in patients with insulin resistance [20]. The glucose content of the RPMI-1640 was 11 mM, to which glucose was added to achieve a final glucose concentration of 25 mM in the experiments. Insulin or additional glucose was added either at the same time as TNF-α or LPS, or 3 h prior to addition of TNF-α or LPS. Culture media were collected and centrifuged (14,000*g* for 5 min at room temperature). The supernatants were frozen at −20 °C until assayed. The cells were washed once with cold PBS and their contents extracted in a detergent-based buffer, as follows. A 0.5 mL aliquot of RIPA buffer (150 mM NaCl/1% NP40/0.5% Tween 20/0.1% SDS/1 mM EDTA), containing 10 μL/2 mL Protease Inhibitor Cocktail III (Calbiochem, San Diego, CA) was added to each well. The culture wells with RIPA buffer were incubated on ice for 10 min, with occasional mixing. The cells were then scraped free in a 96-well plate with a cell scraper and the well contents suspension were centrifuged 14,000*g* for 5 min at 4 °C [21]. The resulting supernatants (RIPA extracts, 2 million cells in 0.2 mL) were collected and stored at −20 °C. All experiments were carried out in 3–5 replicate wells and repeated at least three times.

2.5. In vivo study

Wild type C57BL6/J male mice (control group) and TNF-α null male mice (9–12 weeks old) received an intra-peritoneal injection

of either 100 μL of normal endotoxin-free saline (control) or 100 μg of LPS (in 100 μL saline). One hour after injection, blood was obtained by cardiac puncture and stored at 4 °C overnight. The following day, the serum was collected by centrifugation at 5000*g* for 10 min at room temperature, then transferred to a fresh tube and stored at −20 °C until assay.

2.6. Activin A ELISA

Dimeric activin A was measured by a two-site ELISA (Oxford Bio-innovations, Cherwell, Oxfordshire, UK), using human recombinant activin A as standard [22], which has 100% peptide sequence identity with mouse activin A. This assay measures both free and follistatin-bound activin A dimers and has no significant cross-reaction with other isoforms of activin or with TGF-β. The mean sensitivity was <12 pg/mL. The mean intra- and inter-assay coefficients of variation were 5.0% and 6.7%, respectively.

2.7. Statistical analysis

Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for the graphing and statistical analyses. Two-way Analyses of Variance and *t*-tests were used to determine significant differences between groups, in conjunction with a Student–Newman-Keuls multiple comparisons of means test. All data are presented as mean ± standard deviation (sd) with *p* values less than 0.05 defined as a significantly different.

3. Results

3.1. Regulation of activin A release and content by TNF-α and LPS in cultured BMNPs

In three individual preparations of freshly isolated bone marrow cells, intracellular activin A concentrations of BMNPs were 140.7 ± 29.4, approximately 7-fold higher than that of the bone marrow mononuclear cells (21.1 ± 12.8). Isolated BMNPs released activin A into the culture medium continuously over 12 h of culture and TNF-α stimulated the secretion of activin A approximately 2-fold within the first hour (Fig. 1A). The increase in secretion of activin A by the neutrophils stimulated with TNF-α was not related to an increase in activin A production, as total (secreted plus cell content) activin A in the cultures was not affected (Fig. 1B and C). Comparison of activin A concentrations within the cells indicated that most TNF-α inducible release (40% of total activin A) occurred within the first hour of culture, rising more slowly to about 60% of total activin A by 12 h of culture. In contrast with TNF-α, LPS had no significant effect on secreted or total activin A. There was a slight (<30%) increase in total activin A in the cultured cells up to 4 h of culture, but this production was not appreciably affected by either LPS or TNF-α. These data indicate that the increased secretion of activin A by murine neutrophils stimulated with TNF-α *in vitro* was not due to production of new protein, but rather release of pre-existing, stored activin A. Moreover, LPS had no effect on activin A secretion or synthesis by BMNPs in culture.

3.2. Regulation of activin A release and content in total and fractionated bone marrow cells

Since activin A release *in vivo* is stimulated by LPS, the ability of LPS to regulate activin A release by isolated murine bone marrow cells was investigated further. One hour after LPS treatment, total bone marrow cells secreted significantly more activin A than untreated cells, while LPS had no effect on secretion by either purified BMNPs or mononuclear cells from the same bone marrow preparation (Fig. 2A). Cell content was unaffected by LPS in any cell type over the same period (Fig 2B), but total activin A was significantly increased by LPS in the total bone marrow cells (Fig. 2C). These data indicate that LPS-induced release of activin A by bone marrow cells involves cooperation and communication between the BMNPs and mononuclear cells, as well as a net increase in activin A production.

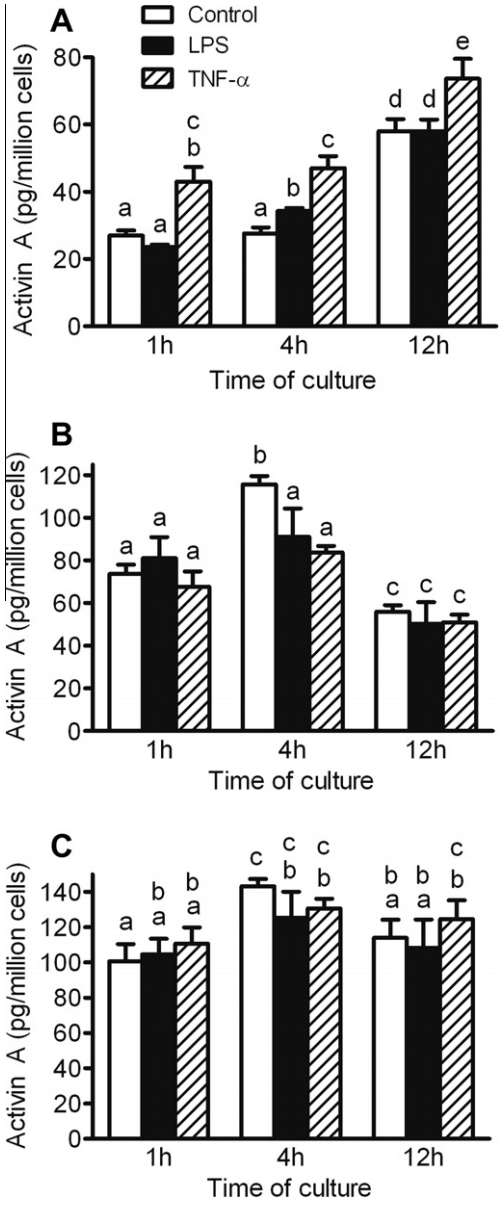

Fig. 1. Time course of activin A secretion (A), cell content (B) in bone marrow neutrophil precursors following treatment with TNF-α (100 ng/mL) or LPS (100 ng/mL). Values are mean ± sd ($n = 3$ wells) from a representative experiment. Values with same letter superscript are not significantly different, $p > 0.05$.

3.3. Response of serum activin A to an LPS challenge in TNF-α knockout mice

LPS treatment *in vivo* stimulated the release of activin A protein into the circulation of both wild-type mice and TNF-α null mice within one hour (Fig. 3). These data establish that TNF-α is not the only regulator of activin A release during inflammation.

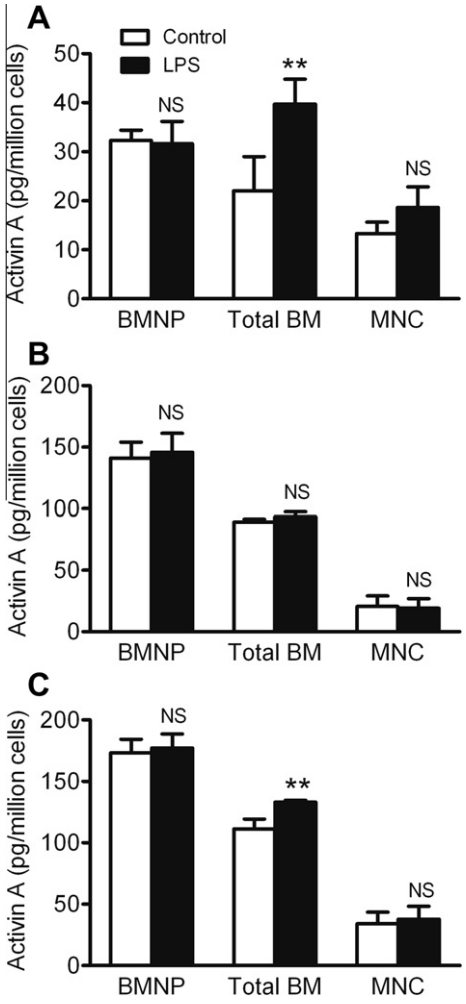

Fig. 2. Activin A secretion (A), cell content (B) and total activin A (C) in bone marrow neutrophil precursors (BMNPs), total bone marrow (BM) derived cells and mononuclear cells (MNC). Cells were cultured for one hour in the presence of LPS (100 ng/mL) or without treatment (control). Values are mean ± sd ($n = 3$ wells) from a representative experiment. $^{**}p < 0.01$, compared with control group. NS: not significantly different from control at $p > 0.05$.

3.4. Regulation of activin A release and content by insulin and glucose in cultured BMNPs

Finally, given the interactive relationship between TNF-α, activin A and neutrophil function in T2D, the ability of glucose and insulin to regulate activin A production by BMNPs was investigated. After pre-treatment of BMNPs for 3 h with insulin, the effect of TNF-α on activin A release was completely blocked (Fig. 4A). By contrast, insulin had no effect on activin A release when added at the same time as TNF-α (data not shown), and insulin had no effect on activin A cell content (Fig. 4B) or total activin A (Fig. 4C) under any conditions investigated. Although, there was evidence that elevated glucose had a slight inhibitory effect on activin A levels in BMNPs based on cell content values (Fig. 4B), this was not reflected in significant changes in activin A secretion (Fig. 4A) or total activin A (Fig. 4C).

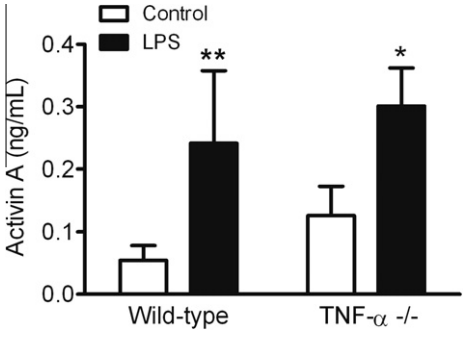

Fig. 3. Activin A concentrations in the serum of wild-type and TNF-α null mice one hour after injection with saline or LPS (dose). Values are mean ± sd ($n = 7$ for wild-type groups and $n = 3$ for TNF-α −/− groups). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group.

4. Discussion and conclusions

In summary, these studies establish that BMNPs from adult mouse bone marrow contain preformed activin A, which they spontaneously secrete in culture, but synthesis of activin A by these cells in culture was comparatively low, leading to a corresponding decline in cell content. Activin A secretion was stimulated (approximately 2-fold) by the inflammatory cytokine TNF-α, and pretreatment of the cells with insulin eliminated the response to TNF-α. In contrast to the direct effect of TNF-α, the effect of LPS appears to be exerted indirectly. Treatment with LPS stimulated

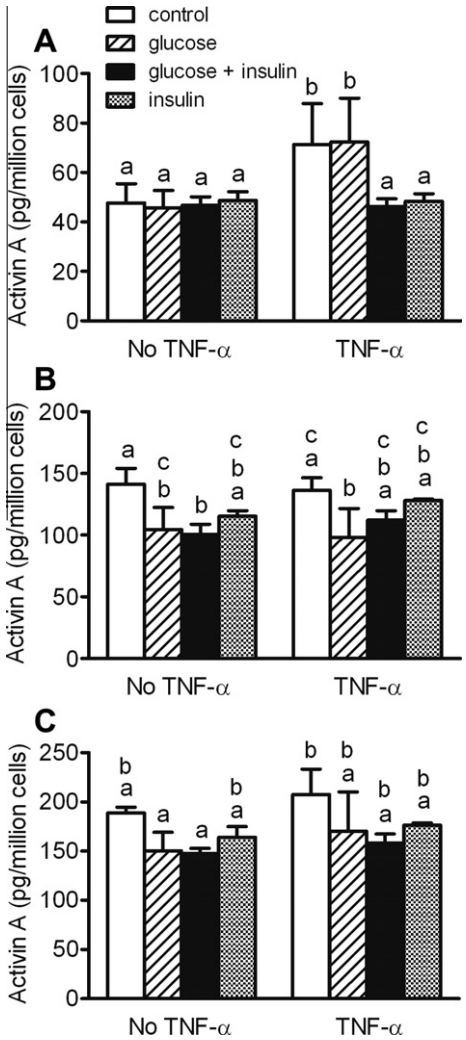

Fig. 4. Activin A secretion (A), cell content (B) and total activin A (C) in bone marrow neutrophil precursors. Following incubation with insulin (0.5 mU/mL) and glucose (25 mM) alone or in combination, cells were stimulated with TNF-α (100 ng/mL), or untreated. Values are mean ± sd ($n = 3$ wells) from a representative experiment. Values with same letter superscript are not significantly different, $p > 0.05$.

activin A release from total bone marrow cells, but not in purified bone marrow neutrophil precursors or mononuclear cells, and LPS treatment of mice stimulated serum levels of activin A in both normal and TNF-α null mice. These results, together with our earlier studies on human blood neutrophils, indicate that neutrophils are an important source of activin A, which can be stimulated to be released by TNF-α, although TNF-α is clearly not the only stimulator of activin A release during inflammation. The fact that LPS cannot stimulate neutrophil activin A release by direct action suggests that activin A release by neutrophils in LPS-induced inflammation involves interactions with other cell types, possibly mediated by TNF-α or other inflammatory mediators.

It is clear that TNF-α, but not LPS, is able to directly stimulate the acute release of activin A by neutrophils from both mouse and human, independent of effects on activin A synthesis by these cells [4]. Neutrophils express TNF-α receptors on the cell surface and many inflammatory agents can affect the expression of TNF-α receptors in this cell type [23]. Given that neutrophils also express the LPS receptor, i.e. toll-like-receptor (TLR) 4, and CD14, the LPS co-receptor [24], and given that these two inflammatory agents act via overlapping, albeit distinct, signalling pathways [25], the failure of LPS to directly stimulate activin A release is surprising. However, expression of TLR4 and CD14 by these cells is relatively low, and neutrophils are much less sensitive than monocytes to LPS stimulation in general [24]. On balance, the data suggest that the effects of TNF-α on activin A release in neutrophils may involve pathways that are not shared with LPS.

However, LPS is able to stimulate release and production of activin A protein in total bone marrow cell cultures, which might be due to the action of molecules produced by the mononuclear cells which can either regulate activin A secretion or potentiate the response of the neutrophil to LPS. Neutrophils respond to several inflammatory agents produced by activated monocytes, including TNF-α, interleukin (IL)-1β and IL-8 [26,27]. The data in the present study indicate that TNF-α is a regulator of activin A release by neutrophils, but further suggest that TNF-α is probably not the main regulator of activin A release during LPS-induced inflammation [3]. This was also indicated by our earlier studies showing that injection of a specific TNF-α receptor antagonist did not affect the release of activin A after LPS treatment in sheep [2]. On the other hand, injection of the activin binding protein, follistatin, reduced the release of TNF-α in LPS-treated mice, which suggests that activin A and TNF-α might also show interactive regulation in causing the release of each other [3].

The ability of insulin to inhibit activin A release induced by TNF-α remains to be explained. Insulin has been implicated in the regulation of neutrophil function previously [28–30]. Inhibition of activin A secretion from neutrophils by insulin could involve effects at the level of the TNF-α receptor, or its downstream signalling pathway, or it could interfere with the mechanisms of activin A release. These data also suggest that a loss of insulin sensitivity by the neutrophils in T2D could lead to an increase in activin A levels and inflammation, thereby exacerbating the disease. It has been demonstrated that neutrophils express insulin receptors and in T2D, neutrophil function is positively associated with insulin sensitivity [16,31]. It appears that the relative roles of TNF-α, insulin and activin A in controlling inflammation during T2D may be complex and further studies on this interaction, and the specific mechanisms involved, would definitely be valuable. Finally, these data provide further evidence that T2D is, in fact, a chronic inflammatory disease.

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Chapter V - General Discussion and Conclusions

Overall, the studies described in this Thesis have made significant contributions to understanding the release of activin and follistatin from various tissues, and their roles in inflammatory and metabolic disorders, such as insulin resistance and T2D. The data indicated that the bone marrow is the major source of the activin A release into the circulation, especially during systemic inflammation, although other tissues are also involved. Further exploration was made of which cell type in the bone marrow was responsible for this release of activin A, and it was confirmed that bone marrow-derived neutrophils are a major source of activin A that is released by inflammatory stimuli, most notably TNF-α. It appeared that the neutrophils released from the bone marrow might carry activin A to various tissues, particularly the lungs, during acute inflammation. Additionally, it was determined that neither activin A, nor activin B, can be used as independent indicators of T2D. However, they do show a positive correlation with this disease and clinical indicator of its severity. It was also determined that the release of activin A by mouse neutrophils induced by TNF-α is inhibited by insulin, but not by glucose, indicating a novel linkage between the neutrophils, inflammation and insulin deregulation, involving activin A.

V.1 The Source of Activin A and Follistatin – Role of the Neutrophils

Many tissues have been found to contain activin A and follistatin on an individual basis or in a particular setting (Meunier et al. 1988; Tanimoto et al. 1992; Tuuri et al. 1994). However, it has not been clear which tissues are the major sources of these proteins, and which contribute to the significant amounts of these proteins in the circulation even under normal conditions, Increasingly, it is evident that activin A is elevated in many inflammatory disorders and plays an important role in acute inflammation, in particular.

The circulating level of activin A was correlated with negative patient outcomes in clinical septicaemia (Michel et al. 2003), and in a mouse model of severe endotoxemia, pre-treatment by injection to raise circulating levels of the activin-binding protein, follistatin, could improve the survival rate of the mice suffering from septic shock (Jones et al. 2007). Determining the origin of the activin A and follistatin during systemic inflammation would critically advance the knowledge base for future studies on the role of activin A in inflammation, and the potential for follistatin to act as a specific therapeutic agent for controlling activin A and its consequences in inflammation.

In chapter II, it was shown that all of the tissues of male mice examined express activin β_A-subunit mRNA, although the level of expression was highly variable (more than 100-fold), with liver showing the highest level of expression. There is no clear data to determine the half-life of activin β_A-subunit mRNA, but it would appear that the mRNA is relatively long-lived compared to other cytokines. This was indicated by the fact that the mRNA levels in the liver were not stimulated by LPS treatment and remained relatively unchanged over a period of 5h in the presence of the transcriptional inhibitor, actinomycin D, in contrast to the response of TNF-α.

In contrast to the wide variation in activin β_A-subunit mRNA expression, most tissues contained similar levels of activin A protein to each other, with the exception of bone marrow which contained several times higher activin A concentrations. This was also true when activin A was calculated as a ratio with follistatin in the various tissues, and is consistent with previously published studies showing that bone marrow stromal cells and myeloid cells produce activin A *in vitro* and *in vivo* (Takahashi et al. 1990; Uchimaru et al. 1995; Yamashita et al. 1992). One hour following treatment with LPS, there was a

significant decrease in activin A in the bone marrow, suggestive of its release from bone marrow into the circulation. Immunohistochemistry of bone marrow cells localised the activin A to several cell types, including the neutrophil precursors. Following LPS treatment, there was a significant reduction in the number of activin A-immunopositive cells and neutrophil precursors in the bone marrow. Immunohistochemistry of the lungs from mice treated with LPS indicated that the neutrophils that accumulated in this tissue were also immunopositive for activin A. These data support the hypothesis that the bone marrow is a major contributor to activin A protein in the circulation, and that neutrophils are a major source of this activin A during acute inflammation. There are no published data suggestive of the source of activin A in chronic inflammation and circulating neutrophils are not always elevated in chronic inflammatory conditions, such as rheumatoid arthritis or systemic lupus erythematosus (Starkebaum 2002).

The immunohistochemical data suggest that activin A is stored in granules within the neutrophil cytoplasm. Although the nature and type of these granules were not determined, it was further noted that the activin A tended to be distributed in a preferentially perinuclear location. Release of the stored activin occurred spontaneously in culture, but this was further stimulated by TNF-α. In human neutrophils, the stimulatory effect of TNF-α on activin A release was shown to be dependent upon signalling via a p38 MAP kinase-mediated pathway (Chen et al. 2011). However, the baseline release of activin A in culture is more likely to be attributable to the spontaneous apoptosis that neutrophils undergo when removed from the bone marrow environment (da Silva et al. 1994).

The role of the neutrophil as a significant source of activin A during inflammation was not evident prior to commencement of these studies. It was known that bone marrow

monocytes and stromal cells are able to secrete activin A following stimulation with TNF-α, LPS or IL-1α and that activin A is important for the regulation of various bone marrow cell types (Abe et al. 2002; Aleman-Muench and Soldevila 2012). Previous studies have implicated activin A as a key regulator of B-cell and T-cell development in the bone marrow, able to promote growth and differentiation of multipotent progenitor cells and erythroid precursor cells (Aleman-Muench and Soldevila 2012). In addition, activin A can induce the synthesis of prostanoids in rat bone marrow macrophages and stimulate activation of NF-κB, and phosphorylation of ERK 1/2 and p38 MAP kinase in murine bone marrow macrophages (Aleman-Muench and Soldevila 2012; Nüsing and Barsig 1999).

In chapter IV, bone marrow neutrophils were extracted from adult mice. Basal neutrophil content for activin A protein was relatively high, and was much greater than the activin A concentrations in enriched mononuclear cell fractions, which included both the bone marrow myeloid cells and stromal cells. This was further evidence that neutrophils are a major source of activin A. Of particular interest was the fact that LPS had no effect on the secretion of activin A from bone marrow neutrophils in culture, but TNF-α had the capability to stimulate directly this release. This was consistent with what had been demonstrated in human neutrophils in parallel studies from our laboratory (Chen et al. 2011b). This was also indicative of a close interaction between activin A and TNF-α, as initially suggested by the fact that both TNF-α and activin A increase rapidly within the circulation of mice and sheep following LPS treatment over the same time-course. Moreover, blocking activin A with follistatin prior to activation of an inflammatory cascade induced by LPS resulted in a substantial reduction in the TNF-α peak (Jones et al. 2007). The slightly later production of IL-6 was also reduced, and there was a significant

delay in the release of IL-1β as well. It has been shown *in vitro* that activin A can regulate production of these cytokines and other pro-inflammatory genes by human, rat and mouse monocyte/macrophages (Nüsing and Barsig 1999; Yamashita et al. 1993). These data suggest that TNF-α and activin A may be enhancing the production and secretion of one another during the initial inflammatory response. The lack of effect of LPS on the immediate release of activin A by cultured neutrophils suggests that LPS does not directly stimulate this secretion, even though neutrophils express the TLRs, including TLR4 (Nathan 2006). On the other hand, the rapid release of activin A does not depend on TNF-α exclusively, since activin A was stimulated by LPS in mice lacking TNF-α, and injection of a specific TNF-α receptor antagonist did not affect the release of activin A after LPS treatment in sheep (Jones et al. 2004a). These findings indicate that activin A is a pro-inflammatory cytokine in acute LPS-induced inflammation *in vivo*.

However, activin A can reduce inflammation, as well as the production of TNF-α, in macrophages under different circumstances (Aleman-Muench and Soldevila 2012; Cuschieri et al. 2008). Insulin has been shown to inhibit macrophage activation by decreasing the phosphorylation of ERK1/2, p38 and JNK MAP kinases, and the activation of NF-κB, by maintaining intracellular SH2-containing inositol 5′-phosphatase levels, a regulator of the PI3K/Akt pathway (Cuschieri et al. 2008). This effect of insulin was blocked by inhibiting activin A, indicating that activin A is a necessary intermediate in this inhibition. By contrast, insulin had no effect on basal activin A production by murine neutrophils in culture, but prevented the ability of TNF-α to stimulate activin A production by these cells. Altogether, these data indicate that insulin inhibits inflammation and does this, at least in part, by regulating activin A production of myeloid cells. Curiously, the inflammatory role of activin A and the actual effects of insulin on

activin A production appear to depend upon either the activation status of the responding cell, or the type of cell, whether macrophage or neutrophil. This complex interaction between insulin and inflammation, mediated by activin A, clearly requires more study.

V.2 The Regulation of Activin A and Follistatin in Inflammation

The studies in chapter II showed that circulating activin A was elevated after LPS treatment and the levels of activin A displayed a peak at one hour and remained elevated for at least seven hours after treatment. Follistatin also increased in the circulation after LPS treatment, but this was not elevated at one hour and did not reach a peak until the five hour time-point. The initial release of activin A was, therefore, independent of follistatin, suggesting the secretion of activin A that was not bound to follistatin. The release of TNF-α into the circulation coincided with or occurred immediately after the release of activin A, but declined much more rapidly than activin A. A similar profile was observed for IL-1β.

The very early release of activin A suggests two potential mechanisms could be at work: pre-stored activin A protein being released or rapid synthesis of new activin A. Since there was no increase in β_A-subunit mRNA in any tissue at one hour after LPS treatment, this suggested that induction of mRNA was not involved. In order to further examine this hypothesis, the mRNA transcription inhibitor, actinomycin D, and a protein translation inhibitor, cycloheximide, were administered to the mice prior to the LPS injection. The results show that cycloheximide injected one hour before LPS reduced activin A concentrations in the circulation by 50%, but had no effect on follistatin and delayed the peak release of TNF-α by one hour without changing the apparent size of the peak. Injection of actinomycin D had no significant effects on the initial rise in activin A

concentrations after LPS treatment, but caused a gradual increase in activin A in the blood after 3 hours. Actinomycin D had no effect on follistatin release after LPS, but caused both a delay and an increase in the duration of the peak of TNF-α after LPS. These results suggest that newly translated activin A was produced during the early stages of inflammation, but that the newly synthesized activin A protein was not derived from new mRNA transcription. It appears that the very rapid appearance of activin A in the circulation is due to the release of activin A from cells that store this protein, such as the bone marrow-derived neutrophils, and new protein synthesised by tissues with high levels of pre-existing activin β_A-subunit mRNA, such as the liver. These data indicate that the release of activin A is regulated very differently from the regulation of other inflammatory cytokines, such as TNF-α or IL-1β, which are primarily regulated at the transcriptional level following LPS treatment (Falvo et al. 2010; Perez et al. 1999; Rambaldi et al. 1993). These data provide important new information that may lead to novel therapeutic approaches that potentially could be used for controlling acute inflammation by regulating activin A, by inhibiting the translation and release of activin A during acute inflammation.

V.3 The Role of Activin A in Type 2 Diabetes and Metabolic Disease

Inflammatory disorders include pathologies with an infectious basis, such as septicaemia or meningitis, or non-infective diseases, such as rheumatic arthritis (Kowalski et al. 2008). Inflammation is also implicated as an aetiological factor in metabolic disorders, such as insulin resistance and T2D, obesity and hypercholesterolemia (Guest et al. 2008; Jawień 2008). Several inflammatory cytokines and other mediators are involved in the development of T2D and insulin resistance (Kewalramani et al. 2010; Navarro-Gonzalez et al. 2010; Sjöholm and Nyström 2006). A clear involvement of activin A has been

reported in most inflammatory conditions (El-Gendi et al. 2010; Hübner et al. 1997; Kariyawasam et al. 2009), and there is mounting evidence that activin A plays a role in control of glucose metabolism and the development of T2D (Hashimoto and Funaba 2011). It has already been mentioned that insulin plays a role in regulating activin A release from neutrophils and macrophages (Chen et al. 2011b; Cuschieri et al. 2008). Moreover, activin A, activin B and follistatin have been found in pancreatic β islets, which produce insulin to control blood glucose levels (Brown and Schneyer 2010).

In chapter III, the potential role of activins and follistatin in insulin resistance and T2D was investigated. The results of this study showed that activin A, activin B and follistatin levels were not significantly different in a relative small population of group subjects with impaired glucose tolerance and/or impaired fasting glucose, patients with T2D and normal subjects. This indicated that activin A, activin B and follistatin are not independent indicators of T2D or insulin resistance, although it should be noted that the clinical groups were relatively small in size and heterogeneous with respect to subject characteristics. However, there were clear significant positive correlations between activin A and fasting glucose, insulin, glycated haemoglobin (HbA1c) and HOMA-IR, as markers of diabetes. Activin B was positively correlated with fasting glucose, HbA1c and HOMA-IR, while follistatin was positively correlated with HOMA-IR alone. These results suggest that circulating activin A and B levels are positively related to the severity of the T2D. This also indicates that in a more homogeneous subject subset or larger group of patients and controls, significant increases in activin levels would likely be observed in the T2D groups. Indeed, this was confirmed for activin A by a study published while this Thesis was under preparation, in patients with cardiovascular disease and T2D (Ueland et al. 2012).

One possible reason why the activins were not dramatically elevated in T2D might be explained by the fact that activins have complex effects and interactions with insulin and the onset of T2D. On the one hand, activin A is able to impair insulin sensitivity in insulin target tissues, such as the liver, skeletal muscle and white adipose tissue, and cause insulin resistance (Hashimoto and Funaba 2011). On the other hand, activins can also stimulate the differentiation of pancreatic islet β cells, which enables the islet to produce more insulin to promote glucose uptake and control glucose levels (Hashimoto and Funaba 2011).

The connection between T2D and inflammation has also been indicated from the relationship between insulin resistance and inflammation signalling, specifically TLR4 signalling. It has been established that TLR4 expression and its signalling and functional activation were increased in recently diagnosed T2D (Dasu et al. 2010). Both *in vitro* and *in viv*o studies have shown that TLR4 signalling is involved in the development of insulin resistance (Shi et al. 2006). Free fatty acids, which are elevated in obesity and usually in T2D, are able to interact with TLR4, to induce pro-inflammatory cytokine expression in macrophages, adipocytes, and liver (Phillips et al. 2009; Shi et al. 2006). As a result, TLR4 signalling seems to be needed for a component of insulin resistance induced by free fatty acids in adipocytes and *in vivo* after lipid infusion and high-fat diets (Shi et al. 2006).

Apart from the evidence discussed above, which shows obesity is important for the inflammatory features of T2D and insulin resistance, hypercholesterolemia might provide another link between activin A and T2D. Recent studies show obesity creates a low-grade systemic inflammatory condition (Fain 2006; Hardy et al. 2011). This process involves

up-regulation of systemic immunity, and is characterized clinically by insulin resistance and the metabolic syndrome (Jawień 2008). On the other hand, cholesterol can suppress cellular TGF-β responsiveness, by suppressing Smad-2 phosphorylation and PAI-1 expression, while statins, which are medications that can lower cholesterol, can reverse these effects (Chen et al. 2007). Activins can also stimulate the expression of Smad-2 and PAI-1 (Hariharan and Pillai 2008) and PAI-1 expression has been implicated in insulin resistance (Hashimoto and Funaba 2011). In our clinical data, blood triglyceride and cholesterol levels were also measured. Although the results did not show a significant correlation between activin A and lipid profile, there was a negative correlation between follistatin and high density lipoprotein (HDL) in normal patients and a positive correlation between follistatin and triglyceride in the insulin resistance group. Infiltration of pro-inflammatory M1 macrophages in adipose tissue contributes to insulin resistance and onset of T2D, while anti-inflammatory M2 macrophages might help to improve insulin resistance (Hashimoto and Funaba 2011). It is therefore significant that administration of activin A can promote a switch in macrophage phenotype from the M1 phenotype to the M2 phenotype (Hashimoto and Funaba 2011).

It has been demonstrated that neutrophils express insulin receptors and, in T2D, neutrophil function is positively associated with insulin sensitivity (Moreno-Navarrete et al. 2009; Walrand et al. 2006). Inhibition of activin A secretion from neutrophils by insulin could involve effects at the level of the TNF-α receptor, or its downstream signalling pathway, or it could interfere with the mechanisms of activin A release. These data also suggest that a loss of insulin sensitivity by the neutrophils in T2D could lead to an increase in activin A levels and inflammation, thereby exacerbating the disease.

V.4 Clinical Significance of these Studies

Overall, these studies extend our understanding of the role of activins in inflammatory disorders, such as endotoxaemia and T2D, and have identified the neutrophils as a major source of this cytokine. A previous study from this laboratory showed that injection of follistatin is able to alter the profiles of key inflammatory cytokines, such as, TNF-α, IL-1β and IL-6 after LPS stimulation and increase the survival rate of mice with endotoxaemia (Jones et al. 2007). In a rat model of bleomycin-induced pulmonary fibrosis associated with elevated activin, injection of follistatin reduced the number of neutrophils in bronchoalveolar lavage fluid and attenuated TGF-β-induced fibroblast activation and improved the levels of pulmonary fibrosis (Aoki et al. 2005). Therefore, in a clinical setting, follistatin might target neutrophils in treating acute inflammation featuring elevations in activin A. Furthermore, it was demonstrated that TNF-α stimulates release of activin A from neutrophils. These data, along with the study showing that anti-TNF-α treatment is able to improve the glucose profile of T2D patients (Gupta-Ganguli et al. 2011), suggest the possibility, which would need to be evaluated, that anti-TNF-α treatment might have a therapeutic effect in some inflammatory disorders by inhibiting TNF-α-induced activin A elevations.

Although the activins and follistatin are not independent predictors of T2D or insulin resistance, it appears that the activins may be indicators of the severity of the disease, since they are positively correlated with the glucose and the insulin resistance profile, such as fasting glucose, HbA_1c, HOMA-IR. Clinically, doctors assess the past 12 weeks glucose control with HbA_1c and assess the degree of insulin resistance by with HOMA-IR. Since activin A or B are positively correlated with fasting glucose, HbA_1c and HOMA-IR, they may also predict not only glucose control conditions, but also the degree of insulin

resistance, and provide the clinician with additional information to identify the severity of the disease.

In summary, this study widens our understanding of the roles of activin family members in acute and chronic inflammatory disorders. Additionally, the study provides more understanding on the mechanism of the aetiology and development of metabolic disorders, such as T2D, obesity and hypercholesterolemia. The results also suggest that regulation of neutrophil activin A release by insulin may play a role in the inflammation that is associated with T2D, providing new directions for future management of this disease.

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