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

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ABSTRACT

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

1. Introduction

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

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

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infarction is associated with elevated serum activin A levels which correlate with peak serum creatine kinase concentrations [12]. In animal studies, activin A gene expression has been found to be up-regulated following myocardial ischaemia–reperfusion and it has also been implicated in myocardial remodelling in congestive heart failure [13,14]. As activation of TLR4 pathway may be one of the key features of ischaemia–reperfusion induced inflammation, these studies raise the possibility that activin A could be involved in the downstream inflammatory pathway.

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

In the present study, we show that activin A gene and protein expression is increased following myocardial ischaemia–reperfusion in mice and that follistatin administration reduces myocardial infarct size *in vivo*. Furthermore, follistatin reduces murine cardiomyocyte damage and improves mitochondrial membrane potential (MMP) *in vitro*, without affecting ischaemia–reperfusion induced reactive oxygen species (ROS) production.

2. Materials and methods

2.1. Reagents

Bovine follistatin was purified in our laboratory from ovarian follicular fluid, as previously described [19]. Bovine, human and mouse follistatin have 97% identity at the amino acid level. Human recombinant activin A (100% amino acid identity with mouse activin A; R&D systems, Minneapolis, MN, USA) was a generous gift from Dr. Craig Harrison (Prince Henry's Institute, Victoria, Australia).

2.2. Experimental animals

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

2.3. In situ murine ischaemia–reperfusion model

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

2.4. Determination of myocardial area at risk and infarct size

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

2.5. Activin A ELISA

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

2.6. Follistatin radioimmunoassay (RIA)

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

2.7. Quantitative reverse transcriptase-PCR (QRT-PCR)

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