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Activated protein C attenuates acute ischaemia reperfusion injury in skeletal muscle

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Abstract

Activated protein C (APC) is an endogenous anti-coagulant with anti-inflammatory properties. The purpose of the present study was to evaluate the effects of activated protein C in the setting of skeletal muscle ischaemia reperfusion injury (IRI). IRI was induced in rats by applying rubber bands above the levels of the greater trochanters bilaterally for a period of 2 h followed by 12 h reperfusion. Treatment groups received either equal volumes of normal saline or activated protein C prior to tourniquet release. Following 12 h reperfusion, muscle function was assessed electrophysiologically by electrical field stimulation. The animals were then sacrificed and skeletal muscle harvested for evaluation.

Activated protein C significantly attenuated skeletal muscle reperfusion injury as shown by reduced myeloperoxidase content, wet to dry ratio and electrical properties of skeletal muscle. Further in vitro work was carried out on neutrophils isolated from healthy volunteers to determine the direct effect of APC on neutrophil function. The effects of APC on TNF-α stimulated neutrophils were examined by measuring CD18 expression as well as reactive oxygen species generation. The in vitro work demonstrated a reduction in CD18 expression and reactive oxygen species generation.

We conclude that activated protein C may have a protective role in the setting of skeletal muscle ischaemia reperfusion injury and that this is in part mediated by a direct inhibitory effect on neutrophil activation.

Keywords: Neutrophil activation; Endothelial dysfunction; Reactive oxygen species

Introduction

Reperfusion of an acutely ischaemic limb is associated with both local and systemic pro-inflammatory responses which can be detrimental to either patient or limb survival. The majority of cases of acute limb ischaemia occur secondary to acute arterial embolism, spontaneous thrombosis in an atheromatous artery or failure of an arterial graft. Trauma is also an important cause of an acutely ischaemic limb and modern methods of vascular surgery, fracture fixation and soft tissue reconstruction have improved dramatically the potential for limb salvage in this setting. There are currently no effective interventional strategies for attenuating the

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reperfusion injury that accompanies limb revascularisation because the pharmodynamics or toxicity of certain agents has limited their clinical use. Therefore, the discovery of such an agent that would attenuate the reperfusion injury may also play a role in limiting the development of complications such as Volkmann's ischaemic contracture and posttraumatic compartment syndrome and possibly improve limb survival rates following acute arterial ischaemia and revascularisation.

Much research has focussed on the area of skeletal muscle ischaemia reperfusion injury over the past number of years and while it is not fully understood, an aberrantly activated immune response, characterised by neutrophil-mediated injury is felt to play a central role in this process. The primary target of the reperfusion injury is the microcirculation where the leukocyte–endothelium interaction results in transendothelial migration and tissue injury from the release of reactive oxygen species and elastases [16,17]. Although skeletal muscle has a relatively high tolerance to ischaemia, skeletal muscle dysfunction and infarction are well-recognised complications of the reperfusion injury. The muscle injury is characterised by endothelial damage and permeability oedema, which if untreated, may lead to a compartment syndrome and muscle necrosis. It has previously been shown that depletion of leukocytes has a protective effect on reperfused tissue after an ischaemic insult [10,13].

Human Protein C is a plasma serine protease that plays a well-understood role in maintaining haemostasis. Protein C circulates in the blood in its inactive form. As thrombin is formed, thrombin binds to thrombomodulin on the endothelial cell surface and it is this thrombin–thrombomodulin complex that converts Protein C to its activated form. Activated Protein C (APC) along with its cofactor protein S, functions to block further thrombin formation through a feedback inhibition mechanism by inactivating factors Va and VIIIa [3]. Thrombin as well as being a well known pro-coagulant is also a pro-inflammatory mediator. Thrombin increases surface expression of P-selectin on the endothelium and is also a potent agonist for the synthesis of platelet activating factor [11,18]. Furthermore it has been shown to induce ICAM expression, an important event in leukocyte adhesion [12]. This reinforces the concept that coagulation, particularly thrombin generation, and inflammation are co-ordinately regulated and that the protein C pathway plays a critical role in linking these processes. Activated protein C has been extensively studied in the setting of sepsis and phase III clinical trials (PROWESS) have recently been completed in which APC has been shown to significantly reduce mortality in patients with severe sepsis [1].

This study tested the hypothesis that Activated Protein C attenuates skeletal muscle injury in the setting of ischaemia reperfusion injury and whether APC had a direct inhibitory effect on neutrophil activation, an important event in ischaemia reperfusion injury.

Materials and methods

Hindlimb ischaemia and reperfusion model

Adult male Sprague–Dawley rats (Biological Services Unit, University College Cork, Ireland) weighing 300–350 g were used in all experiments. A rubber band model of tourniquet hindlimb ischaemia and reperfusion was employed. In brief, under 60 mg/kg intraperitoneal (ip) thiopentone sodium anaesthetic, bilateral rubber bands were applied above the greater trochanters to interrupt the arterial blood supply to the hindlimbs. Preliminary experiments employing several animals confirmed global ischaemia and subsequent reperfusion with the aid of a laser Doppler blood flow monitor probe (MBF 3D; Moor Instruments, Axminster, UK). After 2 h, the rubber bands were removed initiating hindlimb reperfusion. Animals used in this study were maintained in accordance with the guidelines of the Cruelty of Animals Act, 1876, of the Department of Health, Ireland, and those of the European Community Directive (86/609/EC).

Animal group

The animals were randomised ($n = 10$ per group) into three groups. Group A underwent anaesthesia alone as a control for the anaesthesia. Group B underwent an IV injection of normal saline 15 min prior to tourniquet release followed by 12 h reperfusion. Group C underwent a similar volume IV injection of Activated Protein C (100 g/kg) (InnovativeResearch, Southfield, MI 48034, USA) at the same time point. Ischaemia reperfusion was induced by application of rubber bands above the greater trochanters bilaterally. Following 12 h reperfusion, the animals were then sacrificed for tissue harvesting. The extent of skeletal muscle injury was measured by tissue wet to dry ratio, myeloperoxidase content and electrical properties of the muscle.

Functional assessment of tibialis anterior muscle

After 12 h reperfusion, while still under IP anaesthesia, the tibialis anterior muscle of each animal was exposed. The animals were fixed to an external frame in a supine position. A 2–0 silk suture was tied around the distal tendon which was then sectioned and attached to a force transducer (AD Instruments, Europe) to measure the isometric contractile force. The muscle temperature was maintained at 32–33 °C using an over-head heating lamp. The in situ muscle was stimulated directly (0.1 ms duration, 10 V) via two electrodes connected to a stimulator(AD Instruments, Europe). The length of resting muscle was adjusted to produce maximum twitch tension. The isometric twitch contractile properties were determined. Tetanic tension in response to a tetanic electrical stimulus (50 Hz) was then recorded for each muscle. Twitch and tetanic contractions were reported as N/g of muscle weight.

Wet to dry ratio

Sections of gastrocnemius were excised and weighed (wet weight). The muscle was then dried at 60 °C in a convection oven for 72 h and reweighed (dry weight). Wet to dry ratios were calculated and used as an index of oedema formation.

Myeloperoxidase assay

Fresh tissue samples were homogenised in buffer A (0.021% K_2HPO_4, 0.663% KH_2PO_4 and 0.5% hexadecyltrimethyl ammonium bromide in distilled water). The homogenates were freeze-thawed three times and centrifuged at 2000 rpm for 10 min. The supernatant was assayed spectrophotometrically for MPO activity by adding 1 ml of supernatant to 2 ml of freshly prepared solution B (Solution B was prepared by dissolving 0.0105 g K_2HPO_4 and 0.3315 g KH_2PO_4 in 40 ml of