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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

distilled water and adding 5 ml of a 0.017% solution of dianisidine in methanol and 5 ml of 0.006% hydrogen peroxide in distilled water). The change in absorbance with time was then measured at 460 nm. One unit of MPO was defined as that which degraded 1 micromole of hydrogen peroxide at 25 °C and was then calculated per gram of tissue.

Neutrophil isolation

Neutrophils were isolated from the heparinized blood of three healthy human volunteers by density gradient centrifugation using 6% Dextran (mol. wt. 52,000, Sigma, UK) in 0.9% sodium chloride for 40 min at 22 °C, centrifugation in Ficoll-paque (Pharmacia LKB Biotechnology, Piscataway, NJ) at 300*g* for 30 min to pellet granulocytes and remaining erythrocytes and centrifugation of the resuspended pellet over an 81% ISOTONIC Percoll (Sigma, UK) gradient at 350*g* for 15 min to pellet erythrocytes. The diffuse layer at the interface containing neutrophils was then harvested, washed, resuspended in medium and counted. Cell viability was >97% as determined by trypan blue exclusion, and neutrophil purity was 95% as determined by Rapi-Diff II (DiaChem, Lancashire, UK) staining. Purified neutrophils were suspended in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate at a final concentration of 1×10^6/ml.

FACS analysis of integrin expression

Following isolation of PMN from healthy volunteers, neutrophils were incubated with APC at a concentration of 80 nM or an equal volume of RPMI medium for 60 min at 37 °C in a humidified 5% CO_2 environment. The neutrophils were then stimulated with TNF-α at a concentration of 1 ng/ml for a period of 90 min. After this time 150 μL of cell suspension was incubated with 10 μL of fluorescently labelled monoclonal antibody (mAb). We used specific mAb to CD 18 (Becton Dickonson Immunocytometry Systems, San Jose, CA, USA) conjugated with FITC, along with the isotype-matched IgG1 control mAb. Samples were incubated for 30 min at 4 °C after which time fluorescence on flow cytometry was determined using FL-1 for FITC conjugated antibody. All the stimulation conditions were performed in triplicates.

Measurement of reactive oxygen species generation

Intracellular formation of reactive oxygen species in neutrophils was detected by using the fluorescent probe dichlorofluorescein diacetate bis (acetoxymethyl) (DCFH-DA) (Molecular Probe, Eugene, OR). Briefly, neutrophils were treated with APC (80 nM) and stimulated with TNF-α at a final concentration of 1 ng/ml, and then washed twice with PBS. Cells were loaded with 20 μM DCFH-DA and incubated in a water bath for 10 min. The measurement of intracellular reactive oxygen species was performed on a FACScan flow cytometer (Becton Dickinson) for detecting the log of the mean channel fluorescence intensity with an acquisition of FL1. The minimum number of 5000 events was collected and analysed on the software CellQuest.

Data analysis

All data are presented as mean values ± standard deviation of the mean. Statistical analysis was determined by one way analysis of variance (ANOVA) with post-hoc Tukey test analysis. Differences were considered significant at $p < 0.05$.

Results

Effect of ischaemia reperfusion on skeletal muscle function

Muscle contractile function was measured after 12 h reperfusion and expressed as the peak tension achieved for each muscle for both twitch and tetanic contractions. IRI impaired muscle twitch contraction compared with the control group. Treatment with Activated Protein C preserved twitch contraction after IRI (Fig. 1). Muscle tetanic contraction was also significantly reduced by IRI when compared with control and again, this reduction was attenuated with APC treatment (Fig. 2).

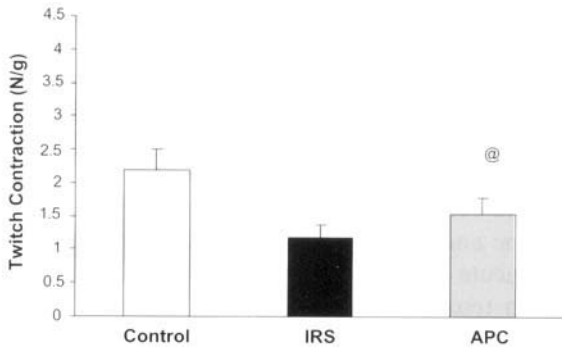

Fig. 1. Effect of APC on mean peak twitch contraction in rats subjected to ischaemia reperfusion injury. Data expressed as mean ± SD for $n = 10$/group. @$p < .022$ vs IRS.

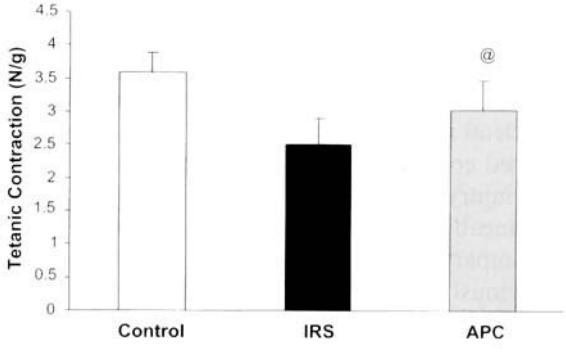

Fig. 2. Effect of APC on mean tetanic contractions in rats subjected to 2 h ischaemia and 12 h reperfusion. Values are expressed as mean ± SD for 10 rats in each group. @$p < .02$ vs IRS.

Tissue oedema

For assessment of tissue oedema, tissue wet/dry ratios were measured. IRI significantly increased skeletal muscle oedema compared with controls. Treatment with APC significantly reduced oedema formation compared to saline treated animals. $p < 0.03$ APC vs IRS (Table 1).

Table 1
Muscle wet to dry ratios

Control group ($n = 10$)	Saline group ($n = 10$)	APC group ($n = 10$)
W/D ratio 3.16 ± .06	3.34 ± .11	3.22 ± .09 @

Mean (SD). @$p < .03$.

Effect of ischaemia reperfusion on muscle neutrophil infiltration

Tissue MPO content was measured as an index of neutrophil sequestration and oxidative damage. Skeletal muscle MPO content was significantly higher after 12 h reperfusion in the saline treated group when compared with controls. Administration of APC significantly inhibited the increase in MPO content after 12 h reperfusion. $p < .023$ APC vs IRS (Fig. 3).

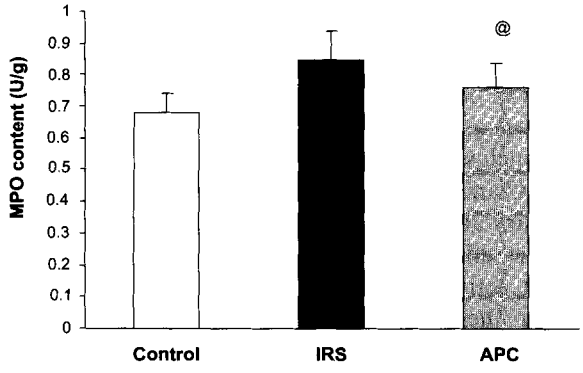

Fig. 3. Effect of IRI on skeletal muscle neutrophil infiltration. Data expressed as mean ± SD in each group. @$p < .023$ vs IRS.

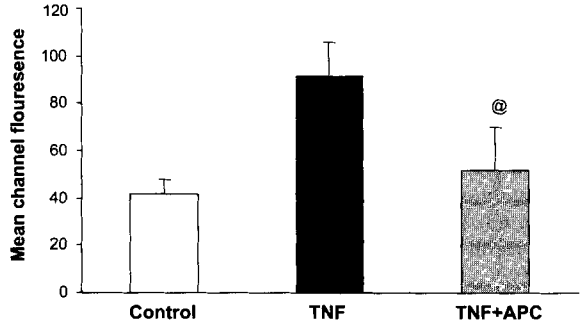

Fig. 4. Effect of APC on CD 18 expression in TNF-α stimulated neutrophils. Data expressed as mean ± SD. @$p < .018$.

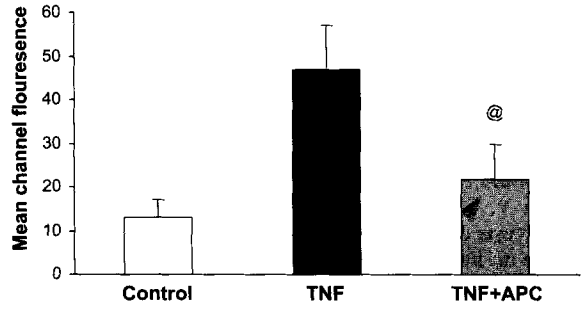

Fig. 5. Effect of APC treatment on reactive oxygen species generation in TNF-α stimulated neutrophils. Data expressed as mean ± SD. @$p < 0.01$.

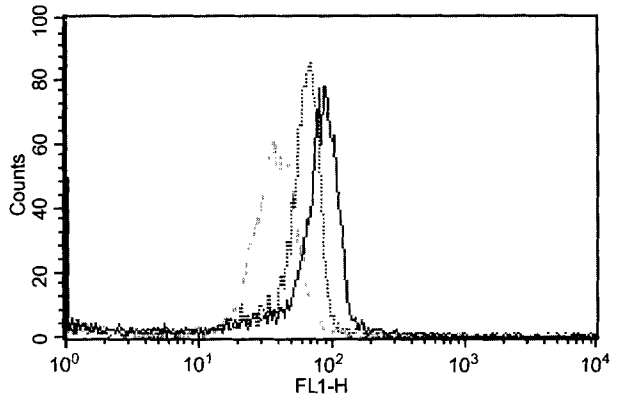

Fig. 6. A flow diagram demonstrating CD 18 expression in unstimulated neutrophils (grey), stimulated neutrophils (black) and stimulated neutrophils treated with APC (dotted line).

Effect of APC treatment on CD18 expression and reactive oxygen species generation in TNF-α stimulated neutrophils

The effect of APC treatment on CD 18 expression and reactive oxygen species generation in stimulated human peripheral blood neutrophils was recorded. APC treatment significantly reduced CD 18 expression and reactive oxygen species generation (Figs. 4–6).

Discussion

Restoration of blood flow to an acutely ischaemic limb initiates a cascade of cellular and biochemical events that result in muscle oedema, necrosis and impaired muscle function. Although the pathophysiology of ischaemia reperfusion injury is complex, there is increasing evidence that neutrophil adhesion to the activated microvascular endothelium is a key step in this process [2]. Activation of circulating PMNs leads to the up-regulation of surface adhesion molecules (CD11a/CD18 and CD11b/CD18) with their adherence to and migration across the endothelium. This results in microvascular injury from the release of reactive oxygen species and elastases, effects potentiated by the delay in inflammatory cell apoptosis that occurs with such activation [14]. In addition, ischaemia reperfusion directly causes dysregulation of endothelial function via accelerated endothelial cell apoptosis that may contribute to the increased microvascular permeability and impairment in barrier function [9].

The present study demonstrates that treatment with APC results in a modest reduction in the tissue oxidative damage and oedema associated with skeletal muscle reperfusion injury. Skeletal muscle injury, measured by oedema and leucosequestration were significantly lower with APC treatment compared to saline treatment. The functional injury induced by IRI was reflected in the

maximum twitch and tetanic contraction forces. The preservation of the contractile properties in the APC treated group further emphasizes the suppression of the inflammatory response provoked by APC. This is the first study that shows that APC may have a protective role in the setting of skeletal muscle ischaemia reperfusion injury. Although there are many studies demonstrating the potent anti-inflammatory properties of APC [5], it could be argued that APC was exerting favourable properties by creating an oncotic gradient and therefore increasing the intravascular volume. To discount this possibility we included a group in our study that was treated with albumin (data not shown) with a similar protein content to APC. As there was no significant improvement in the albumin treated group, this excluded the possibility that APC was exerting a beneficial effect through its oncotic properties.

While activated protein C reduces inflammation by limiting thrombin formation, it also has direct anti-inflammatory properties independent of its anti-coagulant properties. APC has been shown to inhibit the generation of inflammatory cytokines such as TNF-α and to have a selective inhibitory activity on the response of human mononuclear phagocytes to liposaccharide, interferon-γ or phorbol ester [6]. By using gene expression profiling, Joyce et al. examined the direct effects of APC on endothelial cell function and showed that APC suppressed pro-inflammatory pathways and NF-κB-modulated gene expression [8]. Furthermore, APC promoted anti-apoptotic and cell survival pathways in TNF-α stimulated endothelial cells. However in hyper-inflammatory states, protein C is consumed and endothelial damage limits its further activation and function [4]. These findings provide a rationale for the use of activated protein C in certain inflammatory conditions where endothelial cell dysfunction plays a central role.

As already outlined, an aberrantly activated immune response, characterised by neutrophil-mediated injury, appears to play an important role in augmenting muscle injury. While extensive research has been done examining the effects of APC on cellular function, the direct effects of APC on activated neutrophils has not been previously studied. We therefore examined the effects of APC on TNF-α stimulated neutrophils. While many adhesion molecules are required for this process, the integrin Mac-1 (CD11b/CD18) plays a key role in the PMN mediated inflammatory response. In addition to its role in transendothelial migration, Mac-1 also regulates PMN chemotaxis, phagocytosis and respiratory burst [7]. We therefore measured neutrophil CD18 expression, as well as reactive oxygen species generation. Interestingly, APC had no effect on the constitutive expression of CD 18, indicating that it does not suppress baseline neutrophil function. APC did however down-regulate TNF-α stimulated neutrophil activation. This would suggest that APC could have a role to play in the setting of ischaemia reperfusion where neutrophil hyper-activation appears to have a detrimental effect on the host. Further work is required to elucidate the possible mechanisms by which APC attenuates PMN inflammatory response for example, signal transduction pathways such as P38 kinase phosphorylation, transcription factor NF-κB activity and the caspase cascade.

Much of the previous work on skeletal muscle ischaemia reperfusion injury has focussed on the effects of pre-treatment with various agents [15]. While this is important to further our understanding of this complex process, this does not always correlate with the clinical setting where ischaemia is often a sudden unpredictable event. In our experiments, activated protein C was given after the ischaemic insult and a significant reduction in injury was observed, therefore suggesting that it could be used as a therapeutic intervention.

In conclusion, this study demonstrates that activated protein C ameliorates skeletal muscle ischaemia reperfusion injury and our in vitro work demonstrated a direct inhibitory effect on neutrophil activation. We therefore feel that activated protein C may be a potential candidate for therapeutic manipulation of skeletal muscle reperfusion injury in the clinical setting.

References

[1] Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. New Engl J Med 2001;344(10):699–709.
[2] Crinnion JN, Homer-Vanniasankam S, Parkin SM, Gough MJ. Role of neutrophil-endothelial adhesion in skeletal muscle reperfusion injury. Brit J Surg 1996;83:251–4.
[3] Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. J Biol Chem 1989;264:4743–6.
[4] Fisher CJ, Yan SB. Protein C levels as a prognostic indicator of outcome in sepsis and related diseases. Crit Care Med 2000;28: S49–56.
[5] Grey ST, Tsuchida A, Hau H, et al. Inhibition of LPS-induced activation of human monocytes by activated protein C occurs through a novel mechanism. J Leukocyte Biol 1993;114:A487.
[6] Grey ST, Tsuchida A, Hau H. Selective inhibitory effects of the anticoagulant activated protein C on the response of human mononuclear phagocytes to LPS, IFN-gamma or phorbol ester. J Immunol 1994;153:3664–72.
[7] Issekutz AC, Rowter D, Springer TA. Role of ICAM-1 and ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration. J Leukocyte Biol 1999;65:117–26.
[8] Joyce DE, Gelbert L, Ciaccia A, et al. Gene expression profile of antithrombotic protein C defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 2001;276(14 (April 6)): 11199–203.
[9] Kohli V, Selzner M, Madden JF, et al. Endothelial cell and hepatocyte deaths by apoptosis after ischaemia/reperfusion injury in the rat liver. Transplantation 1999;67(8):1099–105.
[10] Korthuis RJ, Grishman MB, Granger DN. Leukocyte depletion attenuates vascular injury in postischaemic skeletal muscle. Am J Physiol 1988;254:H823–7.
[11] Lorant DE, Patel KD, McIntyre TM, et al. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or

thrombin: a juxtacrine system for adhesion and activation of neutrophils. J Cell Biol 1991;115:223–34.
[12] Rahman A, Anwar KN, True AL, Malik AB. Thrombin -induced p65 homodimer binding to downstream NF-kappa B site of the promoter mediates endothelial ICAM-1 expression and neutrophil adhesion. J Immunol 1999;162:5466–76.
[13] Romson JL, Hook BG, Kunkel SL. Reduction of the extent of ischaemia myocardial injury by neutrophil depletion in the dog. Circulation 1983;67:1016.
[14] Sookhai S, Wang JH, McCourt M, et al. A novel therapeutic strategy for attenuating neutrophil-mediated lung injury in vivo. Ann Surg 2002;235(2):285–91.
[15] Wakai A, Winter DC, Street JT, et al. Onosine attenuates tourniquet-induced skeletal muscle reperfusion injury. J Surg Res 2001;99(2):311–5.
[16] Weiser MR, Gibbs SA, Valeri CR, et al. Anti-selectin therapy modifies skeletal muscle ischaemia and reperfusion injury. Shock 1996;5:402–7.
[17] Welbourn CR, Goldman G, Paterson IS. Pathophysiology of ischaemia reperfusion injury: central role of the neutrophil. Brit J Surg 1991;78:651–5.
[18] Zimmerman GA, McIntyre TM, Prescott SM. Thrombin stimulates the adherence of neutrophils to human endothelial cells in vitro. J Clin Invest 1985;76:2235–46.