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Anti-endotoxin Hyperimmune Globulin Attenuates Portal Cytokinaemia, Phagocytic Cell Priming, and Acute Lung Injury after Lower Limb Ischaemia-reperfusion Injury

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Objectives. *Acute limb ischaemia is a common and often lethal clinical event. Reperfusion of an ischaemic limb has been shown to induce a remote gut injury associated with transmigration of endotoxin into the portal and systemic circulation, which in turn has been implicated in the conversion of the sterile inflammatory response to a sepsis syndrome, after lower torso ischaemia-reperfusion injury. This study tests the hypothesis that an anti-endotoxin hyperimmune globulin attenuates ischaemia-reperfusion (I/R) associated sepsis syndrome.*
Design. *Prospective, randomised placebo controlled trial, animal experiment.*
Materials and methods. *Experimental porcine model, bilateral hind limb I/R injury, randomised to receive anti-endotoxin hyperimmune globulin or placebo.*
Results. *Bilateral hind limb I/R injury significantly increased intestinal mucosal acidosis, portal endotoxaemia, plasma cytokine (TNF-alpha, IL-6, IL-8) concentrations, circulating phagocytic cell priming and pulmonary leukosequestration, oedema, and capillary-alveolar protein leak. Conversely, pigs treated with anti-endotoxin hyperimmune globulin (IgG) 20 mg/kg at onset of reperfusion had significantly reduced portal endotoxaemia, early circulating phagocytic cell priming, plasma cytokinaemia and attenuation of acute lung injury.*
Conclusions. *Endotoxin translocation across a hyperpermeable gut barrier, phagocytic cell priming and cytokinaemia are key events of limb I/R injury induced systemic inflammation and acute lung injury. This study shows that an anti-endotoxin hyperimmune globulin attenuates portal endotoxaemia, which may reduce early phagocytic cell activation, cytokinaemia and ultimately acute lung injury.*

Keywords: Ischaemia-reperfusion injury; Anti-endotoxin antibody; Systemic inflammatory response syndrome; Endotoxin; Acute lung injury (ALI); Acute respiratory distress syndrome (ARDS).

Introduction

Acute limb ischaemia is a common and often lethal clinical event.[1] A national audit conducted by the Vascular Surgical Society of Great Britain and Ireland into outcomes in acute limb ischaemia reported an amputation rate of 16 percent and a mortality rate of 22 percent respectively.[2] Mortality rates in these patients remain high after open or endovascular revascularisation due not only to co-existing cardiovascular disease but also as a result of systemic effects attributable to reperfusion of the ischaemic limb.[3,4] Reperfusion of the ischaemic lower limb initiates the systemic inflammatory response syndrome (SIRS), characterised by pro-inflammatory cytokine production, and increased circulating polymorphonuclear (PMN) leucocyte activation.[5–7] After lower limb ischaemia-reperfusion injury (IRI) pulmonary sequestration of activated neutrophils is followed by acute pulmonary microvascular injury, acute lung injury (ALI) which may progress to acute respiratory distress syndrome (ARDS), with a subsequent high mortality.[8–10]

We and others have previously shown that lower limb ischaemia reperfusion injury is associated with increased intestinal permeability and endotoxaemia in association with systemic inflammation and vital organ dysfunction in models of limb ischaemia-reperfusion

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injury,[11] acute limb ischaemia,[12] and after infra-renal and thoracic aortic aneurysm repair.[13,14] It has been postulated that the intestine is a source of inflammatory propagation in critical illness. Endotoxin, a lipopolysaccharide (LPS) component of the gram-negative bacterial cell wall, has been shown to translocate from the intestinal lumen to the portal circulation, thereby providing a potent stimulus to cytokine production and leukocyte activation.[15,16] The precise role of endotoxin in systemic inflammation and sepsis syndrome after vascular surgery remains controversial.[17,20,21]

This study tests the hypothesis that an anti-endotoxin hyperimmune globulin may attenuate systemic inflammation and acute lung injury after lower limb ischaemia-reperfusion injury.

Methods

Experimental Protocol. The studies described herein were performed in accordance with the Animals (Scientific Procedures) Act 1986, and with approval of our institution's Animals Research Committee.

Experimental Model. In these series of experiments we used an established large animal model of lower limb ischaemia-reperfusion injury, as previously described by Harkin, *et al*.[7,18] In brief, male white Landrace pigs (28−35 kg; 6−8 weeks old) were sedated with stresnal (Azaperone BP; i.m. 2−3 mg/kg) prior to induction and maintenance of anaesthesia with Sagatal (sodium pentobarbital; i.v. 20 mg/kg and 8 mg/kg/h respectively), titrated to maintain apnoea. A tracheostomy was created by insertion of a No. 7.5 endo-tracheal tube and mechanical ventilation (tidal volume; 15 ml/kg) was instituted to maintain the arterial partial pressure of CO_2 (Pa_{CO2}) at 30−42 mmHg. Hydration was maintained throughout the experiment by infusion of compound sodium lactate (Hartmann's solution; Baxter Healthcare Ltd., UK; 15 ml/kg/h) and body temperature was maintained at 38.2 ± 0.8 °C. The left carotid artery was catheterized (PE-190, Thomas, Philadelphia, PA, USA) for continuous arterial blood pressure monitoring, arterial blood gas sampling, and systemic blood sampling. A balloon-tip pulmonary artery catheter was guided into the pulmonary artery by pressure wave-form analysis, for central venous (CVP), pulmonary artery (MPAP), and intermittent pulmonary arterial wedge (PAWP) pressure monitoring. Mean pressures were determined using calibrated physiological pressure transducers (Model 1280C) driving an amplifier monitor (Model 7835A; Hewlett-Packard, Andover, MA, USA). The left cephalic vein was catheterized (PE-160; Thomas, Philadelphia, PA, USA) for continuous intravenous infusions of sodium pentobarbital (0.06 mg/kg/min) and Compound Sodium Lactate solution (Hartmann's solution, Baxter, UK). A 14-FG silicon urinary catheter was placed in the bladder for urine sampling. A 7-Fr catheter was advanced through the splenic vein to the portal vein for blood sampling. Tonometer's (Tonometrics Inc., Bethesda, Md, USA) were placed in the gastric antrum and sigmoid colon endo-luminally, and terminal ileum via enterotomy. After assuring haemostasis, the laparotomy was closed. Operative procedures took approximately 60 min, after which subjects were stabilised for 30 min, defined by constant heart rate, arterial pressure, end-tidal CO_2.

Experimental Design. Basal physiological values were recorded after the 30-min recovery period. Blood samples were taken from catheters placed in the systemic, portal, hepatic, inferior vena cava sites. Animals were randomised into three groups ($n = 6$ per group). Two groups underwent bilateral external iliac artery occlusion by application of bulldog arterial clamps for 120 min followed by 150 min of reperfusion on release of bulldog clamps. Absence of flow, and restoration when appropriate, was confirmed using an ultra-sonic flow probe (Transonic Systems Inc, USA) applied to the vessel wall. Six pigs were allocated as sham controls and kept for 270 min with recordings as for the experimental animals (see below).

Treatment. Animals undergoing bilateral hind limb ischaemia were randomised to receive treatment with either Anti-endotoxin hyperimmune globulin (Anti-LPS IgG) at 20 mg/kg body weight, or placebo vehicle administered ($n = 6$ per group) by intravenous infusion over 30 min from the beginning of reperfusion. The endotoxin-core hyperimmune IgG immunoglobulin was prepared from pooled plasma from human donors with high tires of cross-reactive IgG antibodies to endotoxin-core (EndoCAb), using a cold-ethanol process followed by treatment at pH 4 in the presence of pepsin and fresh dried for reconstitution, (*a kind gift from Dr G. Robin Barclay, PhD, formerly Edinburgh & SE Scotland Regional Transfusion Centre, Scottish National Blood Transfusion Service, R&D Laboratories, Edinburgh EH1 1EY, Scotland*).

Blood gas measurements. Arterial blood samples were analysed in an automatic blood gas analyser (1304 pH/blood gas analyser, Instrumentation Laboratory, Warrington, UK) without delay. *Fraction inspired Oxygen (FiO_2)*. This was measured in a ventilatory circuit using an Ohmeda 4700 Oxycap Monitor (Ohmeda, 1315 West Century Drive, Louisville CO 80027, USA). *Arterial-alveolar (A-a) gradient*. This was measured using the formula [(A-a) gradient = fraction inspired $O_2 \times 710 - (\text{arterial } pCO_2/0.8) - \text{arterial } pO_2$]. It is a measure of lung function with a large gradient

indicative of impaired oxygen transport and hence lung injury, as previously described.[18]

Tonometry. Using balloon tonometry gut mucosal pH was calculated by substituting the tonometry derived PCO_2 and the simultaneously measured arterial bicarbonate in the Henderson-Hasselbalch equation: $pHi = 6.1 + \log^{10}([HCO_3]/(0.03 \times PCO_2ss))$ (where 6.1 accounts for HCO_3^- pK value at 37 °C), $[HCO_3^-]$ is the measured arterial blood bicarbonate concentration (mM/l); $PCO^2(ss)$ is the steady-state time adjusted PCO_2 (mmHg) of tonometer saline and 0.03 is the CO_2 solubility coefficient in plasma [mM/l/mmHg] at 37 °C, as previously described.[18]

SMA/Aorta Blood Flow Ratio. During laparotomy ultrasonic flow probes (Transonic Systems Inc, USA) were applied atraumatically to the vessel wall at the origin of the superior mesenteric artery (SMA) and infra-renal abdominal aorta. Continuous monitoring of blood flow in litres per minute was recorded during the experimental period allowing a ratio to be calculated of SMA to aortic blood flow.

Intestinal permeability. Intestinal permeability was assessed as previously described by measuring the urinary excretion of lactulose and mannitol administered via oro-gastric tube immediately after induction of general anaesthesia.[18] The concentration of lactulose and mannitol were measured enzymatically by the reduced nicotinamide adenine dinucleotide (phosphate)-linked enzymatic assays, modified from techniques described by Northrop *et al*., and Lunn *et al*., respectively, using a Cobras Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). The amount of lactulose and mannitol excreted was calculated as a percentage of the administered dose and intestinal permeability was expressed as the lactulose: mannitol ratio.

Blood and Tissue sampling. Blood samples and measurements were obtained every 30 min during the experimental period. Blood samples were collected in heparinized (20 U/ml Blood) sterile pyrogen-free tubes and immediately transferred on ice to be spun at 200 g (at 4 °C) for 10 min. Plasma was aliquoted into sterile cryotubes (Nunc, Intermed, Roskidle, Denmark) and stored at −70 °C until time of assay. Immediately after blood sampling, a mid-line thoracotomy was performed and samples of right lung were excised from predetermined sites. Separate samples were either fixed in 4% formalin for histological analysis or immediately snap-frozen in liquid nitrogen and stored at −70 °C until assayed.

Luminol-enhanced chemiluminescence assay. Chemiluminescence was assayed using a luminal-dependent CL assay as previously described.[7] Respiratory burst was triggered by the addition of 100 µl of phorbol 12-myristate 13-acetate (PMA) into assay tubes. Activity is reported as the integral of the CL events registered during the initial 10 min of activation. The degree of respiratory burst activity is dependent on prior priming in-vivo, further by maximally priming some samples with addition of TNF-alpha to the diluted whole blood a ratio could be determined between whole blood priming and whole blood + TNF-alpha, thus comparing in-vivo priming with maximal priming (%max).

Endotoxin. Endotoxin concentration was determined using a quantitative *Limulus amoebocyte lysate (LAL)* chromogenic assay (Quadratech, Epsom, UK.), as previously described.[18] The detection limits of the assay were between 0.1−1000 pg/ml, inter-assay and intra-assay coefficient of variation were less than 10 per cent.

Interleukin-6 Assay. Biologically active IL-6 was measured using a bioassay based on the proliferation of IL-6-dependent B9 hybridoma cells (a generous gift of L. Aarden, Amsterdam), as previously described.[11,18] The concentration of IL-6 was computed from the standard curve. The detection limits of the assay were between 100−5000 pg/ml, and inter-assay and intra-assay coefficients of variation were less than 10 per cent.

Tumour Necrosis Factor(TNF)-alpha Assay. TNF-alpha is measured by MTT tetrazolium cytotoxicity assay, as previously described.[11,18] The concentration of TNF-alpha in each sample was computed from a standard curve. The detection limits of the assay were between 0.02−200 pg/ml, and interassay and intraassay coefficients of variation were less than 10 percent.

Interleukin (IL)-8 and -10 assays. Plasma levels of IL-8 and IL-10 were analysed by sandwich ELISA according to the instructions of the manufacturer (Biosource International Inc., 820 Flynn Road, Camarillo, CA 93012, USA), as previously described.[7] The detection limits of the assay were between 0.1−1000 pg/ml, and inter-assay and intra-assay coefficients of variation were less than 10 per cent.

Measurement of Lung Tissue Myeloperoxidase (MPO). Lung tissue myeloperoxidase activity was measured spectrophotometrically (Beckmann DU-2 spectrophotometer:Beckmann Instruments, Inc., Cedar Grove, N.J.), using the O-dianisidine hydrochloride method in the presence of 0.0005% hydrogen peroxide, as previously described.[11,18] One unit of MPO activity is defined as the amount required to degrade 1 µmol of peroxide per minute at 25 °C.

Lung Tissue wet-to-dry (W/D) weight ratios. W/D ratios of lung and skeletal muscle tissue samples (200−400 mg) were calculated. Each specimen was blotted dry, weighed then placed in a vacuum freeze dryer at −70 °C for 48 h. Specimens were then re-weighed and the wet-to-dry tissue weight ratio was calculated.

Broncho-alveolar lavage (BAL). BAL was performed through the endotracheal tube using a silastic

pulmonary suction catheter at 240 min, into the right lung. All BAL was performed by the same person and catheter placement confirmed on cadaver studies. BAL fluid was centrifuged at 400 g at 4 °C for 10 min, and the supernatant was stored at −20 °C. The BAL protein content was measured in the non-cellular fraction by the bicinchoninic acid (BCA) method.

Statistical Analysis

Summary values are expressed as mean ± SEM or median (interquartile range). One-way repeated measures analysis of variance was used to compare sequential measurements for parametric data. Dunnett's test was used to make further comparisons if analysis of variance revealed significant differences. The control value for Dunnett's test was designated as the last measurement obtained at the end of the baseline period (time 0). Student's t test was used for independent comparisons for parametric data. Kruskall-Wallis H analysis of variance was used in conjunction with Mann-Whitney U test for non-parametric comparisons. A two-tailed p of <0.05 was considered statistically significant. Statistical calculations were performed using SPSS (Version 14.0, Microsoft, Redmond, WA) software.

Results

Physiological and haemodynamic variables

There was no significant change in the mean base line measurements for heart rate (HR), central venous pressure (CVP), or pulmonary capillary wedge pressure (PCWP) throughout the experimental period, suggesting optimal resuscitation. Mean arterial blood pressure (MAP), was significantly increased in both the I/R group and anti-LPS IgG treated I/R group, compared to control, $P < 0.01$, during the ischaemic period (t = 0−120) due to bilateral iliac artery clamping, but returned to baseline levels on clamp release during the reperfusion period, (data not shown).

Effects of Limb Ischaemia-reperfusion on the Gut

Gut tonometry measurement

Temporal changes in gastric mucosal pH (pHigastric), ileal mucosal pH (pHiileal), sigmoid mucosal pH (pHisigmoid), variables at baseline and for each time point during the experiment are shown in Table 1. A significant fall is seen in gastric pHi (t = 210−270), $P < 0.05$, and more notably sigmoid pHi during the reperfusion period (t = 120−270), $P < 0.01$, in the untreated I/R group compared to both control, and this was not prevented by treatment with anti-LPS IgG treated I/R group.

SMA/aorta blood flow ratio

A relative reduction in blood flow to the gut via the SMA was seen in the I/R group during reperfusion (t = 120−270), although this was not significant and was unaltered by treatment with anti-LPS IgG, Table 1.

Table 1. The temporal changes in gastric mucosal pH, ileal mucosal pH, sigmoid mucosal pH, and fractional superior mesenteric artery blood flow in the experimental groups

	Baseline	T = 60	T = 120	T = 150	T = 180	T = 210	T = 240	T = 270
Gastric-pHi								
Control	7.38 ± 0.06	7.25 ± 0.03	7.23 ± 0.04	7.23 ± 0.03	7.21 ± 0.04	7.25 ± 0.03	7.17 ± 0.05	7.16 ± 0.05
I/R	7.25 ± 0.09	7.22 ± 0.11	7.22 ± 0.11	7.15 ± 0.10	7.16 ± 0.10	7.14 ± 0.09	7.07 ± 0.08[A]	7.08 ± 0.09
I/R + anti-LPS IgG	7.35 ± 0.04	7.21 ± 0.04	7.20 ± 0.01	7.16 ± 0.02	7.13 ± 0.02	7.08 ± 0.03[A]	7.06 ± 0.04[A]	7.03 ± 0.04[A]
Ileal-pHi								
Control	7.26 ± 0.04	7.28 ± 0.02	7.23 ± 0.04	7.29 ± 0.01	7.29 ± 0.01	7.23 ± 0.02	7.22 ± 0.03	7.27 ± 0.04
I/R	7.34 ± 0.05	7.26 ± 0.05	7.26 ± 0.02	7.23 ± 0.02	7.24 ± 0.02	7.21 ± 0.02	7.13 ± 0.07	7.21 ± 0.07
I/R + anti-LPS IgG	7.46 ± 0.05	7.37 ± 0.04	7.38 ± 0.03	7.28 ± 0.03	7.23 ± 0.02	7.21 ± 0.02	7.21 ± 0.05	7.22 ± 0.04
Sigmoid-pHi								
Control	7.26 ± 0.04	7.19 ± 0.04	7.18 ± 0.05	7.16 ± 0.02	7.15 ± 0.03	7.17 ± 0.04	7.12 ± 0.04	7.15 ± 0.05
I/R	7.21 ± 0.07	7.10 ± 0.07	7.14 ± 0.04	7.08 ± 0.03	7.11 ± 0.04	7.06 ± 0.05[B]	7.03 ± 0.06[B]	7.02 ± 0.07[B]
I/R + anti-LPS IgG	7.20 ± 0.06	7.12 ± 0.04	7.13 ± 0.05	7.08 ± 0.05[B]	7.06 ± 0.06[B]	7.04 ± 0.06[B]	7.02 ± 0.07[B]	7.05 ± 0.07
SMA/Aorta Blood Flow Ratio (%)								
Control	0.21 ± 0.04	0.27 ± 0.04	0.27 ± 0.05	0.32 ± 0.09	0.32 ± 0.09	0.34 ± 0.09	0.32 ± 0.07	0.32 ± 0.07
I/R	0.17 ± 0.02	0.26 ± 0.03	0.26 ± 0.03	0.20 ± 0.03	0.22 ± 0.04	0.23 ± 0.05	0.26 ± 0.05	0.26 ± 0.06
I/R + anti-LPS IgG	0.19 ± 0.04	0.26 ± 0.06	0.25 ± 0.04	0.21 ± 0.04	0.22 ± 0.03	0.22 ± 0.03	0.22 ± 0.03	0.21 ± 0.03

[A] $P < 0.05$ vs. control group.
[B] $P < 0.01$ vs. control group.

Lactulose/mannitol urinary excretion ratio

The lactulose/mannitol (L/M) urinary excretion ratio was significantly greater in the I/R group, 0.19 ± 0.03, compared to control group, 0.09 ± 0.03, $p < 0.02$, this was not altered by treatment with anti-LPS IgG.

Endotoxaemia

Portal vein endotoxin concentration increased during the reperfusion period in I/R group, 505.70 (413.70–530.70) pg/ml, compared to control group, 18.92 (10.27–59.75), $p < 0.009$, and significantly attenuated in the anti-LPS IgG treated I/R group, 8.49 (7.48–31.25), $p < 0.05$. (Fig. 1A). There was a significant, $p < 0.008$, positive correlation, $r = 0.604$, between plasma portal endotoxin concentration and L/M urinary excretion ratio, Spearman's rank correlation.

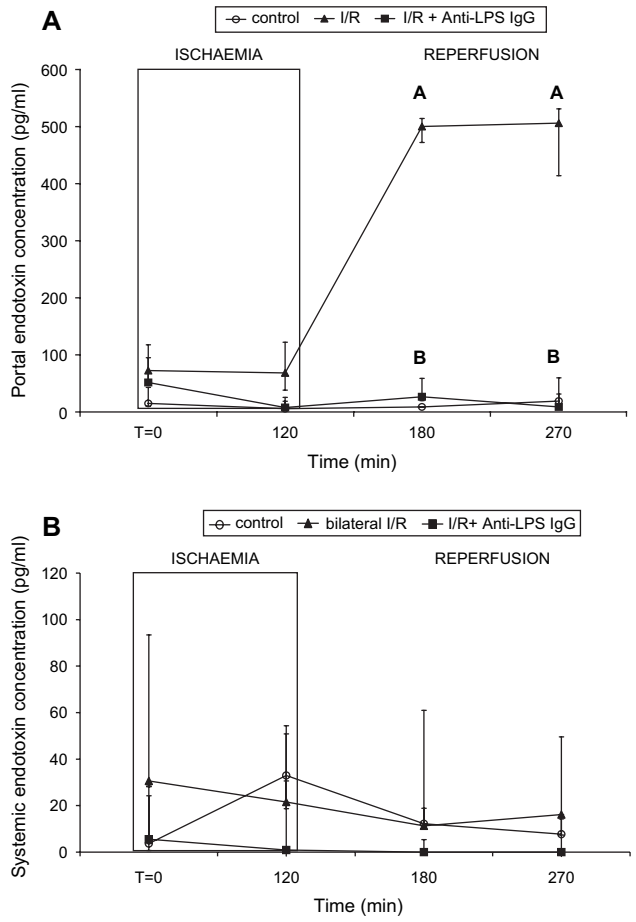

Fig. 1. A: Portal endotoxin (pg/ml) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.009$ compared with control. $^{B}P < 0.05$ compared with I/R group. B: Systemic endotoxin (pg/ml) in control, I/R, and anti-LPS IgG + I/R groups. Data represents mean ± SEM.

Systemic vein endotoxin concentrations were comparible in the I/R and control groups in the reperfusion period. In the I/R group the endotoxin concentration in the systemic circulation fell during reperfusion timepoints t = 180, 11.30 (0.00–61.00), and t = 270, 16.20 (0.00–49.60), compared to the portal vein at t = 180, 500 (472–514.20), $p < 0.01$, and t = 270, 505.70 (413.70–530.70), $p < 0.001$, respectively, (Fig. 1B).

Cytokine Response

Plasma tumour necrosis factor(TNF)-alpha

Portal vein TNF-alpha concentration was significantly elevated after one hour of reperfusion (t = 180) in the I/R group, 229.69 (194.35–274.74) pg/ml, compared to control group, 3.93 (0.0–41.35) pg/ml, and anti-LPS IgG treated I/R group, 20.15 (14.66–27.79), $p < 0.001$. This effect persisted through reperfusion. (Fig. 2A).

Systemic vein TNF-alpha concentration was also significantly greater after one hour of reperfusion (t = 180) in the I/R group, 86.83 (40.79–158.99) pg/ml, compared to control group, 32.67 (0.95–42.82) pg/ml, and anti-LPS IgG treated I/R group, 10.84 (5.78–12.3), $p < 0.05$. In the I/R group significantly less TNF-alpha was detected during reperfusion in the systemic circulation as compared to the portal circulation, $p < 0.01$. (Fig. 2B)

Plasma Interleukin (IL)-6

Portal vein IL-6 concentration was significantly greater after one hour of reperfusion (t = 180) in the I/R group, 1869.0 (1697.0–2090.4) pg/ml, compared to control group, 364.1 (290.3–413.0) pg/ml, $p < 0.001$, and anti-LPS IgG treated I/R group, 1207.0 (1138.3–1428.4), $p < 0.01$. This effect persisted through reperfusion. (Fig. 3A).

Systemic vein IL-6 concentration was significantly greater after one hour of reperfusion (t = 180) in the I/R group, 1726.0(1130.0–2130.0) pg/ml, compared to control group, 303.5(274.4–374.3 pg/ml, $p < 0.003$), and this was not attenuated in the anti-LPS IgG treated I/R group. (Fig. 3B).

Plasma interleukin (IL)-8

Systemic vein IL-8 concentration was significantly elevated after 1 h of reperfusion (t = 180) in the I/R group, 198.1 (116.1–373.9), compared to control group, 49.5 (37.8–73.7), $p < 0.03$, and the anti-LPS IgG treated I/R group, 74.7 (57.6–104.75) pg/ml,

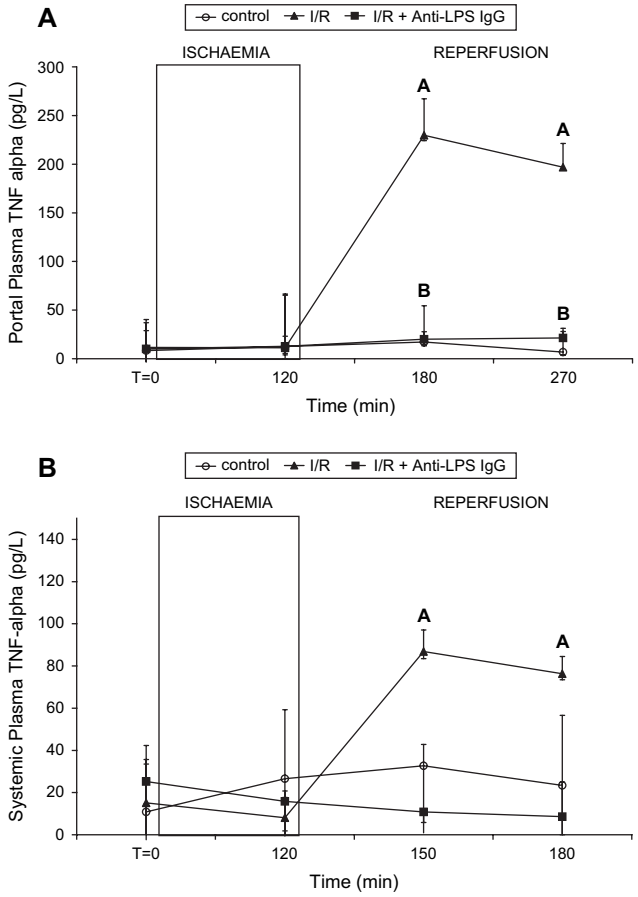

Fig. 2. A: Portal TNF-alpha (pg/L) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.001$ compared with control and anti-LPS IgG + I/R. $^{B}P < 0.001$ compared with I/R group. B: Systemic TNF-alpha (pg/L) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.05$ compared with control and anti-LPS IgG + I/R groups. Data represents mean ± SEM.

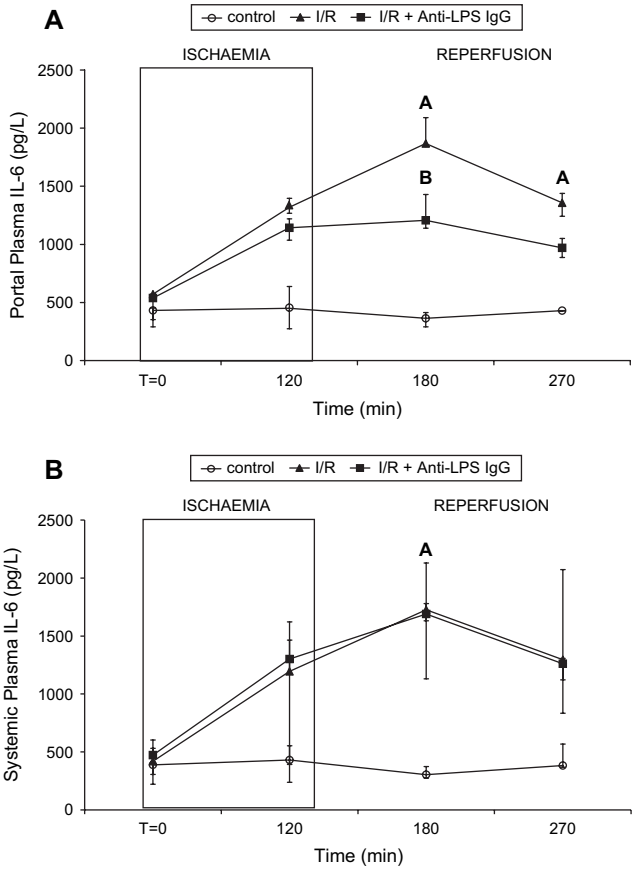

Fig. 3. A: Portal IL-6 (pg/L) in control, I/R, and $rBPI_{21}$ + I/R groups. $^{A}P < 0.001$ compared with control. $^{B}P < 0.01$ compared with I/R group. B: Systemic IL-6 (pg/L) in control, I/R, and anti-LPS IgG groups. $^{A}P < 0.003$ compared with control. Data represents mean ± SEM.

$p < 0.008$. This effect was maintained throughout reperfusion.

Plasma IL-10

Systemic IL-10 concentration was not significantly altered from baseline throughout the experimental period in control, or I/R groups.

Phagocytic Cell Priming for Respiratory Burst Activity

Phagocytic cell priming ratio from whole-blood chemiluminescence assay

Portal vein phagocytic cell priming ratio was significantly greater during early reperfusion (t = 150) in the I/R group, 0.76 ± 0.05, compared to control group, 0.54 ± 0.06, $p < 0.01$, and compared to anti-LPS IgG treated I/R group, 0.62 ± 0.05, $p < 0.05$. After two hours of reperfusion (t = 240) the priming ratio remained significantly greater in the I/R group, 0.81 ± 0.11 compared to control group, 0.24 ± 0.04, $p < 0.001$, and anti-LPS IgG treated group, 0.55 ± 0.08, $p < 0.05$. The priming ratio decreased significantly in the control group from the start (t = 0), 0.59 ± 0.06, to the end (t = 240), 0.24 ± 0.04, $p < 0.005$. Priming increased in the I/R group from t = 0 to t = 240, 0.56 ± 0.20 compared to 0.81 ± 0.11, although this did not reach significance. (Fig. 4A).

Systemic vein phagocytic cell priming ratio was significantly greater during early reperfusion (t = 150) in the I/R group, 1.01 ± 0.08, compared to control group, 0.56 ± 0.11, $p < 0.01$, and compared to anti-LPS IgG treated I/R group, 0.80 ± 0.03, $p < 0.05$. After two hours reperfusion (t = 240) the priming ratio remained significantly greater in the I/R group, 0.86 ± 0.12, compared to control group, 0.26 ± 0.07, $p < 0.001$,

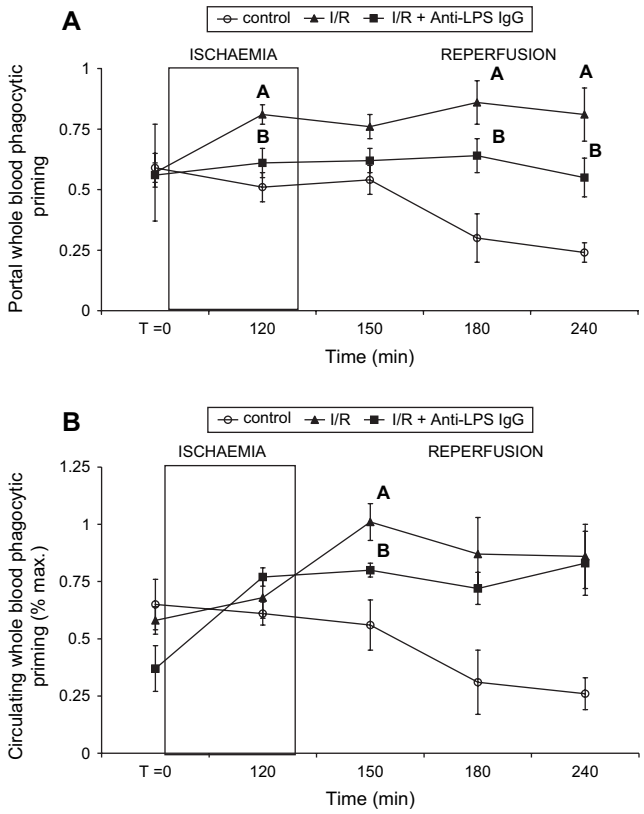

Fig. 4. A: Portal phagocytic cell priming (% max.) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.01$ compared with control. $^{B}P < 0.05$ compared with I/R group. B: Systemic phagocytic cell priming (% max.) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.01$ compared with control. $^{B}P < 0.05$ compared with I/R group.

and this was not attenuated in the anti-LPS IgG treated group, 0.83 ± 0.14. (Fig. 4B).

Whole blood leucocyte counts

There was no difference in total whole blood leucocyte count, nor was there any difference in the neutrophil fraction, between the control group and the I/R group throughout the experimental perior.

Acute Lung Injury

Pulmonary physiology

Arterial partial pressure of oxygen (pO_2 in mmHg) fell significantly during the reperfusion period in the I/R group compared to control group, $P < 0.05$, and attenuated in the anti-LPS IgG treated I/R group, $P < 0.01$. (Fig. 5A).

Pulmonary artery pressure (PAP) was significantly greater during reperfusion in the I/R group compared

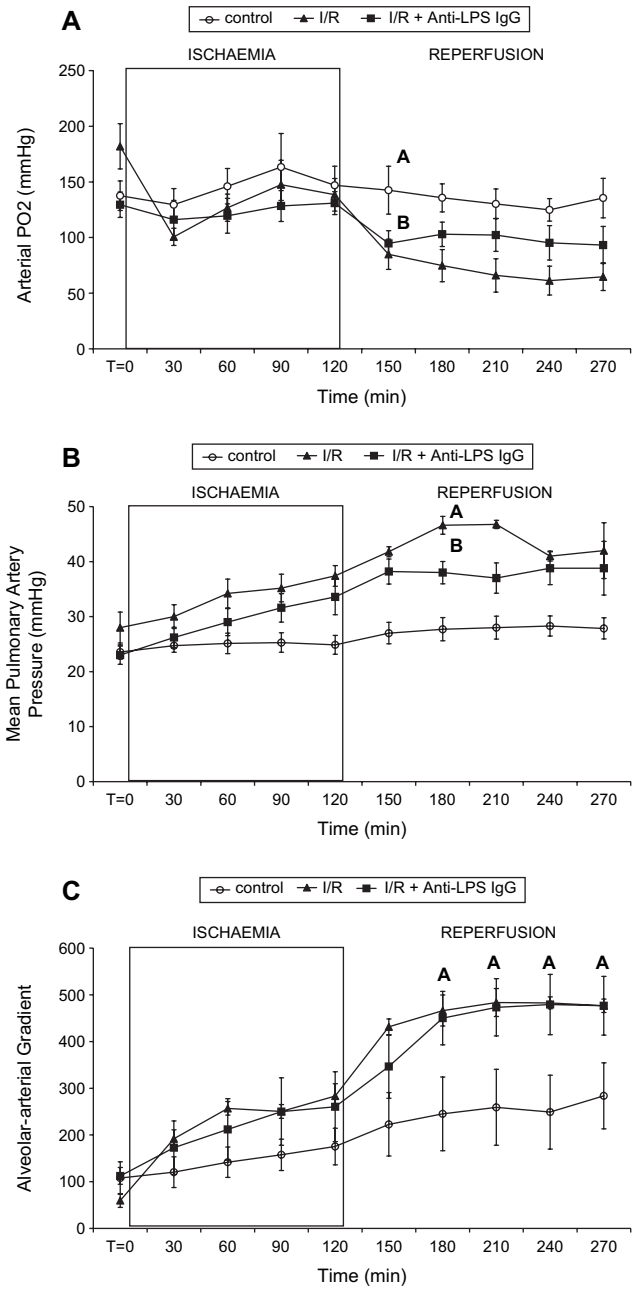

Fig. 5. A: Arterial PO_2 (mmHg) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.05$ compared with control. $^{B}P < 0.01$ compared with I/R. B: Pulmonary artery pressure (PAP) in mmHg for control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.001$ compared with control. $^{B}P < 0.001$ compared with I/R. C: (A-a) gradient in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.05$ compared with control. Data represents mean ± SEM. Repeated measures 2-way Anova (time and group), A: $P < 0.01$, B: $P < 0.01$, C: $P < 0.03$. Post hoc Dunnett's T3 test, A: $P < 0.05$, B: $P < 0.05$, C: $P < 0.01$.

to control group, $p < 0.001$, and significantly attenuated in early reperfusion in the anti-LPS IgG treated I/R group, $p < 0.001$. However, this protective effect was lost in late reperfusion, (Fig. 5B).

Alveolar-arterial (A-a) O_2 gradient values were similar during the ischaemia period but were significantly higher A-a gradient after reperfusion in the I/R group compared to control, $p < 0.05$, suggesting impaired alveolar oxygen transport. This impairment in oxygen transport was not prevented in the anti-LPS IgG treated I/R group, (Fig. 5C).

Lung tissue myeloperoxidase

The lung tissue myeloperoxidase concentration was significantly greater in the I/R group, 33.81 (15.65−52.66) units of absorbance per gram dry tissue, compared to control group, 7.54 (0.97−8.63), $p < 0.005$. This was not attenuated in the anti-LPS IgG treated group, 25.74 (15.89−38.63). (Fig. 6A)

BAL non-cellular fraction protein concentration

The BAL protein concentration was significantly greater after 2 h of reperfusion (t = 240) in the I/R group, 0.72 (0.31−1.60), compared to control group, 0.23 (0.18−0.31), $p < 0.05$. This increase was not attenuated in the anti-LPS IgG treated I/R group, 0.58 (0.44−0.74). (Fig. 6B)

Lung tissue wet-to-dry weight ratio

The lung wet-to-dry tissue weight ratio was significantly greater in the I/R group, 10.13 (7.66−12.66), compared to control group, 5.57 (4.54−7.37), $p < 0.01$. This increase was not attenuated in the anti-LPS IgG treated I/R group, 8.26 (7.06−11.09). (Fig. 6C)

Spearman's rank correlation: correlation MPO and W/D ratio, $P < 0.0001$, r = 0.57; correlation BAL protein and W/D ratio, $P < 0.05$, r = 0.389.

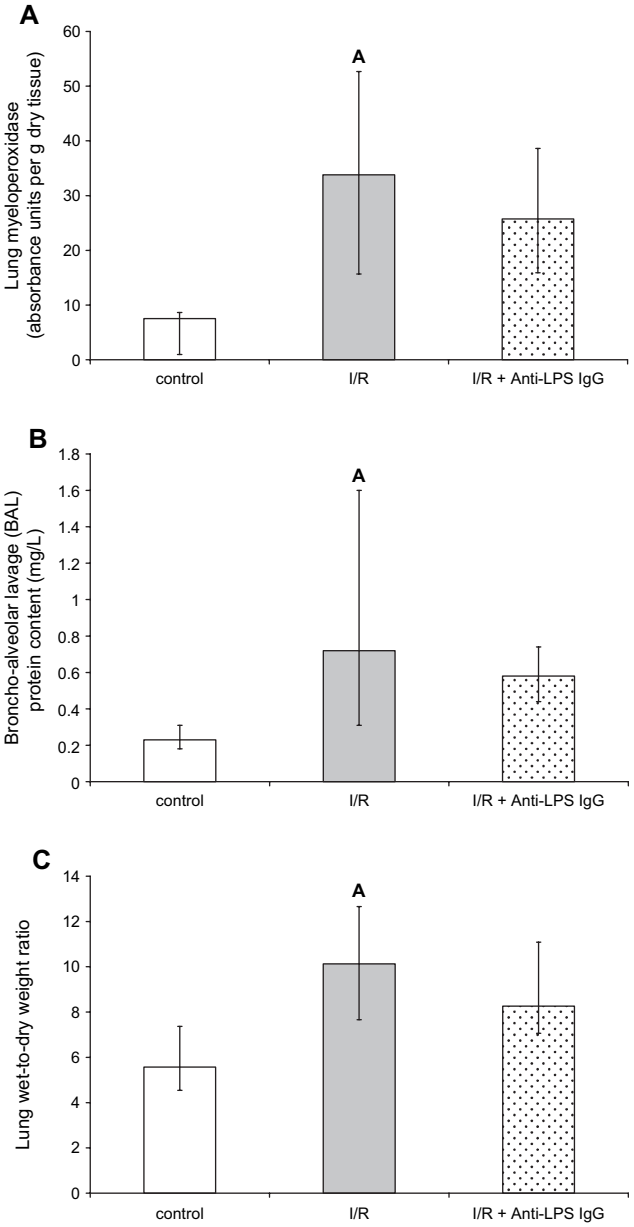

Fig. 6. A: Lung MPO (U/g) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.005$ compared with control. B: BAL protein content (mg/L) in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.05$ compared with control. C: Lung W/D weight ratio in control, I/R, and anti-LPS IgG + I/R groups. $^{A}P < 0.01$ compared with control. Data represents mean ± SEM.

Discussion

In vascular surgery practise patients are commonly exposed to lower limb ischaemia-reperfusion injury during the management of acute limb ischaemia, during revascularization surgery of the lower limb, or during aortic aneurysm repair.[2,3,8] The conversion of lower limb skeletal muscle ischaemia-reperfusion injury into a harmful systemic inflammatory response has been demonstrated in both experimental models[11] and clinical studies.[12] In many patients reperfusion injury may be mild and self-limiting, but reperfusion after prolonged ischaemia of large vascular territories is often lethal.[6] We have shown that severe limb IRI results in remote intestinal injury, and is associated with a significant increase in gastric, ileal, and sigmoid mucosal acidosis.[11,18] Furthermore, that intestinal permeability was increased and associated with a significant increase in portal endotoxin concentration. Treatment with anti-LPS IgG, given post-ischaemia at the start of the reperfusion period, as expected had no effect on intestinal permeability or intestinal mucosal acidosis, but was associated

with a significant reduction in portal endotoxaemia. It is most likely this reduction in portal endotoxin concentration is due to increased leucocyte mediated clearance of endotoxin rather than a reduction in transmigration. Endotoxin, a lipopolysaccharide (LPS) component of the wall of gram-negative bacteria, is undoubtedly a potent stimulus to cytokine production and activation of PMN.[15,22] Impaired splanchnic blood flow has been suggested as a cause of extended gut injury in sepsis.[23] In this model SMA blood flow was reduced as a fraction of total aortic blood flow during reperfusion, although this did not reach significance. Sepsis syndrome remains a leading cause of mortality, and although several classes of LPS-binding compounds have shown promise in basic studies,[24] early clinical trials using anti-LPS monoclonal antibodies have produced equivocal results.[24,25] In this study we have used an anti-endotoxin hyperimmune globulin which has been shown to increase endotoxin binding and clearance by leucocytes,[22,25] due to it's polyclonal nature it is considered much more likely to neutralize LPS derived from the heterogenous bacterial flora of the gut. In this study we have shown increased portal and circulating whole blood phagocytic cell priming after lower limb IRI. Treatment with anti-LPS IgG reduced portal endotoxaemia and early phagocytic cell priming. Priming of PMN to produce reactive oxidant species, by prior exposure to inflammatory stimulus, is thought to be central to the role of PMN-mediated anti-microbial activity and tissue damage in response to a secondary stimulus,[7,19] and is known to be upregulated by exposure to endotoxin.[26] Furthermore, treatment with Anti-LPS IgG and reduction in portal endotoxaemia in this model was associated with significant reduction in pro-inflammatory cytokine concentrations (TNF-alpha and IL-6) in the portal vein. Although it is not possible from these data to confirm the source of these cytokines, greater concentrations in the portal circulation during reperfusion suggest a splanchnic origin. Animal studies of intestinal ischaemia-reperfusion injury,[15,27] and human patients undergoing aneurysm surgery,[28] have shown the portal circulation to be the main source of early pro-inflammatory cytokine production. We did not show significant evidence of systemic endotoxaemia in this study. The hepatic macrophages (Kupffer cells) clear endotoxin and bacteria from the portal circulation during health and disease,[29] although in severe sepsis this system can be overwhelmed or even contribute to systemic inflammation through hepatic cytokine production. Pro-inflammatory cytokines such as TNF-alpha and IL-6 have been shown to be associated with neutrophil priming and delayed apoptosis, and as such may increase the functional activity and longevity of PMN.[30,31] IL-8, a powerful chemoattractant cytokine,[32] was also elevated in this model, and abrogated by treatment with anti-LPS IgG. The balance between pro- and anti-inflammatory stimuli is crucial to the propagation or resolution of inflammation, and beneficial effects of LPS-therapy in these studies may render recoverable a potentially lethal inflammatory syndrome.

In this study the development of remote pulmonary dysfunction was observed only following reperfusion, with development of hypoxaemia, pulmonary hypertension, and impaired oxygen transport. The prevention of this acute pulmonary dysfunction in the anti-LPS IgG treated group suggests a role for endotoxin induced circulating neutrophil activation in the development of this lung injury. Early signs of adult respiratory distress syndrome (ARDS), which carries a high mortality, have been reported within hours of aortic declamping in human abdominal aortic aneurysm repair and animal models of reperfusion injury.[10] The importance of neutrophils in post-reperfusion tissue injury has been confirmed by the attenuation of local limb and remote lung injury in leukopenic models of lower limb ischaemia-reperfusion injury.[19,33] Elevated levels of lung tissue myeloperoxidase, BAL protein concentration, and wet-to-dry weight ratio suggesting a neutrophil mediated acute lung injury characterised by increased microvascular permeability and leukocytic sequestration within the lung. Although there was a reduction in all these measures of injury after treatment with anti-LPS IgG, these were not significant. Despite a reduction in portal endotoxaemia and inflammation in this model measures of lung tissue injury were not attenuated by anti-LPS IgG which may reflect the numerous parallel inflammatory pathways which are activated in reperfusion injury due to vasoactive mediators.[8,10,17,34]

Conclusions

There is persistent evidence supporting a role for gut hyper-permeability and translocation of endotoxin in the propagation of the inflammatory response to significant lower torso ischaemia-reperfusion injury. If gut-endotoxin converts a recoverable inflammatory response to a lethal endotxaemia in a certain subset of patients then anti-endotoxin strategies may still have an important role in the treatment of these patients. In conclusion we have shown that anti-LPS IgG can reduce portal endotoxaemia, cytokinaemia (TNF-alpha, IL-6), neutrophil priming, and remote acute pulmonary dysfunction seen after hind limb ischaemia-reperfusion injury.

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