

Glutamine preconditioning protects against tourniquet-induced local and distant organ injury in a rodent ischemia-reperfusion model

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Introduction Ischemia-reperfusion (IR) injury is a common surgical event, with tourniquet use being a recognized cause in orthopedic surgery. Preconditioning is a highly evolutionarily conserved endogenous protective mechanism, but finding a clinically safe, acceptable method of induction has proven difficult. Glutamine, a known inducer of the heat shock protein response, offers pharmacological modulation of injury through clinically acceptable preconditioning. Our aim was to test the hypothesis that glutamine preconditioning protects against tourniquet-induced regional and remote IR injury in a rodent model.

Animals and methods 40 adult male Sprague-Dawley rats were randomized into 4 groups: control, IR injury, normal saline-pretreated and IR injury, and glutamine-pretreated and IR injury. Pretreated groups received either normal saline or glutamine by intravenous bolus 24 h before injury. A bilateral hindlimb tourniquet model was used. Blood samples were analyzed, bronchioalveolar lavage (BAL) performed, and skeletal muscle and lung harvested for evaluation.

Results The glutamine-pretreated group showed significantly lower muscle myeloperoxidase (MPO) content and creatine kinase levels than the untreated or saline-pretreated injury groups. Lung tissue showed reduced MPO content and a significantly reduced neutrophil count by BAL fluid microscopy.

Interpretation These data suggest that preconditioning with glutamine offers local and distant organ protection in the setting of tourniquet-induced IR injury.

Ischemia-reperfusion (IR) injury is a common surgical event in orthopedics, with tourniquet use being a recognized cause. The pre-emptive induction of intracellular stress proteins by subcritical injury, or preconditioning, is known to protect against subsequent critical injury. Finding a clinically safe and acceptable method of induction has proven difficult. Glutamine, a known inducer of heat shock proteins (HSPs) suggests itself as a safe clinical preconditioning agent (Wischmeyer et al. 2001a). The effect of parenteral glutamine treatment has not been assessed in the setting of limb IR. This study tests the hypothesis that glutamine protects against tourniquet-induced regional and remote organ IR injury.

Reperfusion of the acutely injured limb is a commonly occurring event associated with local and systemic proinflammatory responses, which may account for significant morbidity and mortality (Campbell et al. 1998, Maziak et al. 1998). Pharmacological attenuation of skeletal reperfusion injury may improve limb salvage rates and prevent the local and systemic effects associated with reperfusion following tourniquet ischemia. This may safely permit prolonged tourniquet times in extremity surgery, prevent skeletal muscle ischemia, and reduce the systemic reperfusion injury associated with tourniquet release.

Glutamine is a conditionally essential nutrient during sepsis or trauma (Lacey et al. 1996). It is the most abundant amino acid in plasma and skeletal muscle, but glutamine levels fall dramatically after major injury or infection (Bergstrom et al.

1974). In animal models of sepsis and trauma, glutamine supplementation modifies immune function and improves survival (Zapata-Sirvent et al. 1994, Gianotti et al. 1995, Wischmeyer et al. 2001b).

In vivo administration of glutamine before cardiac surgery has been shown to improve post-reperfusion cardiac function (Wischmeyer et al. 2003). The mechanism of action remains unclear, but the effect may be mediated through improved cardiomyocyte function, enhanced glutathione synthesis, or induction of the heat shock protein response. This effect of glutamine suggests its possible use as a preconditioning agent to induce HSP and improve local and end-organ injury where significant stress is anticipated.

Material and methods

Model of IR-induced lung injury

40 adult male Sprague-Dawley rats (Harlem, U.K.) weighing 300–400 g were used in all experiments. Anesthesia was induced using intraperitoneal sodium pentobarbitone (50 mg/kg) and maintained using inhalational isoflurane and oxygen at 1 L/min. Following anesthesia, the left jugular vein was cannulated using aseptic technique. The model for tourniquet ischemia and reperfusion was prepared as previously described (Kyriakides et al. 2000). Briefly, ischemia was induced for 2 h followed by 2 h of reperfusion. Bilateral rubber bands (latex O-rings) were applied above the greater trochanter for the duration of 2 h. Reperfusion was initiated by removing the bands. Occlusion and reperfusion were confirmed by use of a Doppler ultrasound probe placed distal to the site at which the tourniquet was applied. Control animals received no IR injury. Rats were maintained in a supine position for the duration of the experiment, and covered throughout. Core temperature was monitored using a rectal probe. Animals were maintained on a diet of purified chow pellets and water ad libitum, but fasted for 4 h before their procedure. All animals were killed at the conclusion of 2 h reperfusion. Experimentation was performed under licence from the Department of Health and Children, Republic of Ireland, in accordance with the Cruelty to Animals Act 1876, and with the approval of the local Animal Ethics Committee.

Treatment groups

Animals were randomized at weighing into 4 groups (n = 9–11 per group).

i) The animals in the control group were anaesthetised for the duration of 4 h, but received neither pretreatment nor injury.

ii) The IR injury group received 2 h ischemia and 2 h reperfusion without pretreatment.

iii) The glutamine pretreatment group received glutamine (Sigma-Aldrich Co, St Louis, MO) at 0.5 g/kg as a 4% solution in 0.9% saline.

iv) The saline pretreatment group received a corresponding volume of drug vehicle (normal saline) without glutamine.

Fluids were administered via the lateral tail vein line by slow infusion over 30 min, 24 h prior to IR injury.

Injury parameters

Blood samples. Immediately after anesthesia, and following ischemia and reperfusion, blood samples were taken from the jugular line for biochemical determination of liver function (bilirubin, alanine transaminase, alkaline phosphatase), renal function (urea and creatinine), and muscle trauma (creatine kinase).

Neutrophil infiltration. Following the reperfusion period, a tracheostomy was performed using a 16-gauge intravenous cannula for the purpose of bronchioalveolar lavage (BAL). Following sternotomy the left main hilum was clamped, and lavage was performed by infiltrating the right lung with 2 mL saline, which was repeated 3 times, and the fluid was collected in a tube with EDTA. Diff-Quick staining of Cytospin preparations (see below) (Dade Behring, Marburg, Germany) was used for morphological examination of neutrophils in this study. Nuclei stain blue and cytoplasm stains a lighter color of blue. 200 µL of the BAL fluid was pipetted into a disposable cytospin chamber (Cell-Path plc, town, UK). Disposable filters (Shandon, Ireland) were placed between the chamber and the slide. Samples were centrifuged at 1,000 rpm for 5 min (Shandon Cytospin) and the cells were dried in air for 1 h. The slides were then immersed in 3 solutions for 10 sec each. The solutions used for staining the neutrophils were as follows. Solution I: fixing solution containing 1.8 mg/L triaryl methane in methyl alcohol. Solution II: xanthene dye

(1.25 g/L in distilled water). Solution III: azure A, methyl blue, monobasic potassium phosphate, and dibasic sodium phosphate (Accra Labs, town, UK). The samples were simultaneously assessed by two observers in blinded fashion. Neutrophils were identified, counted in 5 high-power fields, and the average was taken.

Myeloperoxidase assay. Myeloperoxidase (MPO) is a haem-containing enzyme found in neutrophils, which can be used as an indirect measure of tissue neutrophil infiltration. Tissue (lung and muscle) MPO content was determined using a modification of the previously described method (McCormick et al. 2003). Briefly, for muscle a sample of left gastrocnemius was dissected and weighed. For lung samples, the right ventricle was cannulated with a 25-G needle and the right pulmonary hilum clamped, after which the pulmonary vasculature of the left lung was washed out with 50 mL of normal saline. A section of left lung was removed and weighed. Samples were then homogenized in solution B (10 mL) containing 0.021% (w/v) K_2HPO_4 , 0.663% (w/v) KH_2PO_4 and 0.5% hexadecyl trimethyl ammonium bromide (HTAB) (all obtained from Sigma-Aldrich) in distilled water. The homogenates were freeze-thawed twice and centrifuged at 2,000 rpm for 10 min. The supernatants were collected and assayed spectrophotometrically for MPO activity by adding 0.1 mL of supernatant to 2.9 mL of freshly prepared solution C. Solution C was prepared by dissolving 0.0105 g K_2HPO_4 and 0.3315 g KH_2PO_4 in 40 mL 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 was monitored every minute for 10 min, at a fixed wavelength of 460 nm (CPU 8720 UV/VIS scanning spectrophotometer; Philips, Eindhoven, the Netherlands). 1 unit of MPO was defined as the amount required to degrade 1 micromole of peroxide/min at 25°C, and was calculated per gram of tissue using the following formula: MPO per gram of tissue = (highest absorbance/10)/weight of tissue used/0.0113.

Tissue edema. The extent of lung and skeletal muscle edema was measured as previously described (Barry et al. 1996, Yoshidome et al. 1999). Briefly, immediately after dissection, samples of fresh lung and muscle tissue from the right

lower lobe of lung and left gastrocnemius, respectively, were weighed and placed in a drying oven at 55°C until a constant weight was obtained. Lung and muscle edema is represented by an increase in the wet-dry ratio.

Western immunoblot for detection of HSP expression. Following intravascular washout, a section of left lung was harvested and snap-frozen in liquid nitrogen. Similarly, samples of muscle tissue were harvested and frozen in liquid nitrogen until further analysis. Prior to analysis the sample was thawed, then homogenized in cold phosphate-buffered saline at 4°C (Ultra-Turrax T8; IKA Labortechnik, Gottingen, Germany) on ice, and transferred to eppendorf tubes (Sarstedt, Wexford, Ireland). Homogenized tissues were centrifuged at 4,100 g for 30 min at 4°C. The supernatants were then collected and the concentration of protein was assayed.

Using the technique previously described (Wischmeyer et al. 2001a), western immunoblot was performed using antibody to HSP70 (HSP72) (mouse monoclonal IgG1, cat. SPA-810; Stressgen, Victoria, BC) as primary antibody (10 µg of antibody in 10 mL of 1% milk powder (Marvel) in PBS-Tween). This mixture was added to the membranes and left for 2 h at room temperature with shaking. Alkaline phosphatase-conjugated goat anti-mouse IgG1 (Serotec, Oxford, UK) was used as secondary antibody (1.5 µL of antibody in 10 mL of 1% milk powder (Marvel) in PBS-Tween), and was incubated with the membranes for 1 h at room temperature with shaking. The membranes were washed 3 times for 5 min each and HSP70 (HSP72) was visualized using bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma-Aldrich): 67 µL of NBT and 33 µL of BCIP in 10 mL of substrate buffer containing 100 mM Tris HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$. The membranes were then inserted into a plastic bag and exposed to X-ray film (CEA or Fuji) in a dark room and developed. Exposure times varied from a few seconds to 30 min depending on the protein concentration and method used.

Statistics

Data are represented as mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Fisher's least-

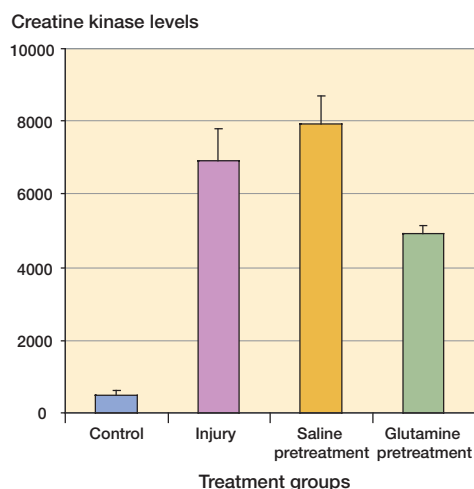


Figure 1. Creatine kinase levels following reperfusion.

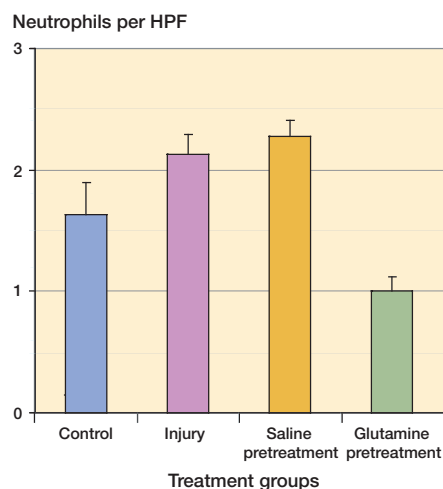


Figure 2. Numbers of bronchioalveolar lavage neutrophils per high-powered field (HPF) by microscopy following reperfusion.

significant difference (LSD) method. A p -value of < 0.05 was considered statistically significant.

Results

No effect of pretreatment on renal or hepatic biochemical markers

Although pretreatment with normal saline and glutamine led to lower urea and creatinine levels than measured in the untreated groups, glutamine pretreatment had no statistically significant effect on renal and hepatic biochemical markers in the blood.

Muscle damage attenuated in the glutamine-pretreated group

Creatine kinase levels in the glutamine pretreated group were significantly lower following ischemia than in the group pretreated with saline ($p = 0.02$) and, following reperfusion, were significantly lower than both the normal saline-pretreated group ($p = 0.001$) and the injury-alone group ($p = 0.02$) (Figure 1). Myeloperoxidase levels in the glutamine-pretreated group were significantly reduced in muscle samples compared to equivalent samples from the injury group ($p = 0.042$).

Pretreatment with glutamine attenuated lung parenchymal damage

In lung tissue, levels of myeloperoxidase were

reduced, but not significantly so ($p = 0.2$). However, measurement of the number of neutrophils in the bronchioalveolar lavage fluid revealed that pretreatment with glutamine significantly reduced neutrophil infiltration into parenchymal tissue compared to the normal saline pre-treated group ($p < 0.001$), the injury-alone group ($p < 0.001$), and even the uninjured control group ($p = 0.02$) (Figure 2).

Wet-dry ratios not convincing

For either lung or muscle specimens, the wet-dry tissue ratios were unaffected by pretreatment with glutamine.

HSP 70 (HSP72) was detected only in glutamine-pretreated tissue

Samples of lung and muscle tissue from all groups were tested for expression of HSP70 (HSP72). Only the samples from the glutamine-pretreated group showed upregulation of the protein, and in this muscle group muscle tissue showed higher levels of upregulation than lung as determined by densitometry of the radiographs of the membranes.

Discussion

Ischemia-reperfusion (IR) injury is a common

surgical event, with tourniquet use being a recognized cause. Tourniquet-induced skeletal muscle ischemia reperfusion is associated with local and systemic proinflammatory responses (Kyriakides et al. 2000). Modulation of this proinflammatory response is difficult, as the precise mechanism by which it occurs has not been fully elucidated. However, a key component in the pathogenesis of reperfusion syndrome is the upregulation of surface adhesion molecules on the vascular endothelium (Stokes et al. 1996) and their subsequent interaction with the activated neutrophils (Hernandez et al. 1987, Granger et al. 1989, Wakai et al. 2001a). Transendothelial migration of neutrophils, with release of reactive oxygen species and cytokines, causes further damage to the injured tissue (Welbourn et al. 1991). There are two main components to the reperfusion syndrome: the local component that causes an exacerbation of the regional ischemic damage, and the systemic sequelae that may cause secondary organ failure remote from the site of ischemia. Resulting pulmonary microvascular permeability can lead to ARDS, while both renal and cardiac injuries can lead to other severe and often fatal complications (Chaudry et al. 1981, Fitzpatrick and Karmazyn 1984).

Our study shows that a clinically relevant pharmacological agent can afford protection to lung and muscle tissue when given as a single intravenous bolus prior to a planned ischemia-reperfusion injury. Agents such as HMG-CoA reductase inhibitors (Dillon et al. 2006), inosine (Wakai et al. 2001b), hypertonic saline (Shields et al. 2003), corticosteroids (Bushell et al. 1996), and vitamin C (Kearns et al. 2001) have recently shown encouraging *in vivo* results when given as a single prophylactic dose prior to a planned ischemia reperfusion injury. To our knowledge, however, no effective prophylactic intervention strategies currently exist to modulate clinically relevant reperfusion injury and its sequelae.

Preconditioning is the phenomenon whereby cells and tissues that are exposed to a sublethal insult are then protected from subsequently lethal insults. It has huge potential as a therapeutic approach for attenuation of planned injuries. Physical strategies include ischemic, hypothermic, and hyperthermic preconditioning. The legion of pharmacological agents investigated has not yet led to any finding

of the “silver bullet”—the discovery of a form of treatment that has effective protective qualities and yet is free from side effects, is noninjurious, inexpensive, and clinically applicable. The potential use of such an agent in tourniquet surgery would be significant, offering a method of (1) increasing the maximum safe length of continuous tourniquet use, while (2) minimizing the local trauma, and (3) reducing the local and systemic effects of reperfusion injury.

This amino acid has a central role in numerous cellular functions. Its metabolic fate in the cell varies depending on the organ system, but it is involved in nitrogen transport, cellular redox control, protein and nucleotide biosynthesis, and it is a source of ATP (Labow and Souba 2000). Although it is the most abundant amino acid in times of health, its levels fall precipitously during times of catabolic stress. Glutamine-supplemented total parenteral nutrition (TPN) has been shown to improve outcome parameters in a range of patient groups (Ziegler et al. 1992, O’Riordain et al. 1994, Griffiths et al. 1997, Houdijk et al. 1998, Morlion et al. 1998).

One of the most basic mechanisms of cellular protection involves the expression of a highly conserved family of essential proteins known as the heat shock proteins or stress proteins. The expression of these proteins after sublethal insults can induce “stress tolerance” and protect against subsequent potentially lethal stresses. Experimental data have shown that the pre-induction of HSPs can provide marked protection against many forms of cellular injury, including ischemia and reperfusion, shock, and lung injury (Wischmeyer 2002). There are *in vivo* data showing that glutamine administration can enhance stress-induced HSP expression and improve outcome following a range of stressful stimuli, including sepsis (Ryan et al. 1992, Ribeiro et al. 1994, Villar et al. 1994), lung injury (Villar et al. 1993), transplantation injury (Perdrizet et al. 1994), and cardiac reperfusion injury (Hayashi et al. 2002). Studies have also shown that glutamine, when given in a range of doses, does not lead to any clinical signs of toxicity—nor to any accumulation of the glutamine metabolites NH_4Cl and glutamate (Wischmeyer et al. 2001a).

In our study, only the group of rats pretreated with glutamine underwent an upregulation of HSP70

(HSP72) expression, and in both lung and muscle tissue. This implicates enhanced HSP72 expression as a potential mechanism for attenuating the expression of MPO in muscle and lung tissue and for reducing the levels of creatine kinase released following both the ischemia and the reperfusion phases of the experiment. Similarly, the reduction in capillary dysfunction and vascular permeability and consequent reduction in neutrophils found in the bronchioalveolar lavage of the glutamine-pretreated group is suggestive of HSP-induced lung protection.

It is interesting that glutamine pretreatment did not seem to afford any attenuation of the reperfusion “hit”, as measured by renal and hepatic biochemistry. Although HSP72 has been induced in the kidney and liver (Wischmeyer et al. 2001a), preinduction of this protein has proven highly difficult to replicate in a clinically acceptable manner. It is possible that the design of this study was not conducive to upregulation of HSP72, given the moderate dose of glutamine given (0.5 g/kg) and the relatively short—although intense—insult provided. Alternatively, the excretive functions of both kidney and liver may mean that these organs have an endogenous protection of their own.

Neutrophil-mediated lung injury is implicated in adult respiratory distress syndrome (ARDS), possibly through the capillary dysfunction of the pulmonary vascular epithelium, which is involved in the imbalance of post-injury immunodysfunction (Moore 1998). Although very early in the pathway to ARDS, it is interesting to note that modulation of this neutrophil response is achieved with glutamine preconditioning. Whether this hinders the organism from mounting a normal physiological response to trauma is debatable, but given the mounting benefits of glutamine therapy seen from studies on other animal trauma models looking at longer endpoints (Wischmeyer et al. 2001b, 2003), this would seem unlikely.

In summary, we have found that preconditioning with a single bolus of the amino acid glutamine prior to a planned tourniquet injury affords both local and distal protection. Glutamine pretreatment affords significant protection to muscle tissue distal to the injury site, and the lungs are also protected, thus reducing the neutrophil-mediated insult from the reperfusion. Upregulation of HSP72 is associ-

ated with this protection mechanism. Our findings have important implications for all branches of surgery where ischemic injury is planned or anticipated, but especially for orthopedic surgery where use of the tourniquet is routine for a range of elective interventions.

Contributions of authors

CGM, DCW, DJB-H: study concept and design. CGM, GC: acquisition of data. CGM: analysis and interpretation of data. CGM, DCW: writing of manuscript. DJB-H: supervision.

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