Protective Effect of Focal Adhesion Kinase against Skeletal Muscle Reperfusion Injury after Acute Limb Ischemia

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WHAT THIS PAPER ADDS

Activation of focal adhesion kinase (FAK) is known to protect heart muscle against ischemia reperfusion (IR) injury. It is shown that IR-induced rhabdomyolysis, macrophage infiltration, and apoptosis of skeletal muscle are fiber type-specific phenomena graded by mitochondria reserves. Moreover, over expression of FAK protected fast skeletal muscle against IR injury by exploiting these reserves, possibly resulting in reduced tissue loss during IR. These findings may lead to novel therapeutic approaches to acute limb ischemia.

Objectives: In cardiac muscle, ischemia reperfusion (IR) injury is attenuated by mitochondrial function, which may be upregulated by focal adhesion kinase (FAK). The aim of this study was to determine whether increased FAK levels reduced rhabdomyolysis in skeletal muscle too.

Material and methods: In a translational in vivo experiment, rat lower limbs were subjected to 4 hours of ischemia followed by 24 or 72 hours of reperfusion. FAK expression was stimulated 7 days before (via somatic transfection with pCMV-driven FAK expression plasmid) and outcomes were measured against non-transfected and empty transfected controls. Slow oxidative (i.e., mitochondria-rich) and fast glycolytic (i.e., mitochondria-poor) type muscles were analyzed separately regarding rhabdomyolysis, apoptosis, and inflammation. Severity of IR injury was assessed using paired non-ischemic controls.

Results: After 24 hours of reperfusion, marked rhabdomyolysis was found in non-transfected and empty plasmid-transfected fast-type glycolytic muscle, tibialis anterior. Prior transfection enhanced FAK concentration significantly ($p = 0.01$). Concomitantly, levels of BAX, promoting mitochondrial transition pores, were reduced sixfold ($p = 0.02$) together with a blunted inflammation ($p = 0.01$) and reduced rhabdomyolysis ($p = 0.003$). Slow oxidative muscle, m. soleus, reacted differently: although apoptosis was detectable after IR, rhabdomyolysis did not appear before 72 hours of reperfusion; and FAK levels were not enhanced in ischemic muscle despite transfection ($p = 0.66$).

Conclusions: IR-induced skeletal muscle rhabdomyolysis is a fiber type-specific phenomenon that appears to be modulated by mitochondria reserves. Stimulation of FAK may exploit these reserves constituting a potential therapeutic approach to reduce tissue loss following acute limb IR in fast-type muscle.

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INTRODUCTION

Acute limb ischemia is among the most common peripheral vascular emergencies. It threatens tissue viability even after a relatively short insult by ischemia reperfusion (IR) associated inflammation and rhabdomyolysis. Apoptotic cell death is a programmed, energy dependent process, which is believed to play a pivotal role in the tissue response to IR. A variety of death signals including calcium overload may trigger apoptosis or inflammation via transition pore-mediated destruction of mitochondria (Fig. 1A). Bcl-2 family molecules, such as BAX and Bc-2, which either promote or inhibit transition pore opening, exert a critical role on whether a cell will live or die. The involvement of
A the Buchenhofer path effect of RISK activation and net mitochondrial biogenesis have been confirmed before.14,15

The aim of the present experimental pilot study was to determine whether FAK expression was modulated by IR and whether the enhancement of FAK expression had an impact on apoptosis and inflammation in IR-injured skeletal muscle.

METHODS

A reality driven rodent model of acute hind limb ischemia16 was used to test whether enhanced FAK expression protected peripheral skeletal muscle from IR tissue injury. The hypothesis was that protective FAK effects should be most marked in muscle fibers with low mitochondrial content, because of their larger reserves towards an oxidative shift.17 As in humans, rat fast-type glycolytic muscles (e.g., tibialis anterior and gastrocnemius muscles) have a lower mitochondrial content than slow-type oxidative muscles (i.e., soleus muscle).18

IR injury was compared across muscle phenotypes (i.e., gastrocnemius vs. soleus muscle) and the effect of enhanced FAK expression was evaluated. For gene transfer, the tibialis anterior was used as fast-type muscle because of its preferred in vivo accessibility as described before.14,15 A paired design was adopted to differentiate local IR injury and protective effects from systemic reactions. Therefore, contralateral limbs served as intra-individual controls whereas external controls were subjected to ischemia only (n = 2) or sham treatment (n = 4). Fig. 1B summarizes the experiment.


Figure 1. (A) Mechanisms of ischemia reperfusion damage and salvage and experimental protocol. RISK refers to a group of pro-survival protein kinases (FAK, AKT, and p70S6K) that confer muscle protection. During the first few minutes of reperfusion, a cellular increase in Ca° occurs which can be partially buffered by mitochondria. At a critical threshold, however, opening of permeability transition pores leads to cell death, either through energy dependent apoptosis, being fuelled by mitochondrial ATP production, or through necrosis. Fast-type muscle fibers have a lower mitochondrial content than slow-type muscle fibers and are more susceptible to IR injury. Endpoints, which were assessed using markers, are underlined and the factors being modulated experimentally are highlighted in color. (B) Experimental design. IR injury was evaluated following 4 hours of ischemia and 24 hours or 72 hours of reperfusion (n = 6 animals). Contralateral limbs served as paired controls, as represented by the double headed arrow. External controls were subjected to ischemia only (n = 2) or sham-treatment (n = 4). The effects following transfection with expression plasmid for FAK (pCMV-FAK) were compared with empty plasmid transfection (pCMV) (n = 13 animals). AKT = serine/threonine protein kinase AKT; BAX = apoptosis regulator BAX; Bcl-2 = apoptosis regulator Bcl-2; FAK = focal adhesion kinase; IR = ischemia reperfusion; MTP = mitochondria transition pore; p70S6K = ribosomal S6kinase; RISK = reperfusion induced salvage kinase. Note: The concept of ischemia and reperfusion injury was modified from refs 4,9,14.
Ethics and funding

All experiments were carried out in accordance with Principles of Laboratory Animal Care (National Institutes of Health publication 86–23, 1985) and after approval of the animal protection and ethics commission of the Canton of Bern, Switzerland. The study was supported by the European Society for Vascular Surgery (ESVS) 2013 research grant.

Ischemia reperfusion

Adult male Wistar rats (mean body weight 390 ± 58 g) were put under inhalation anesthesia (3–5% isoflurane) and placed in supine position on a heating pad. A controlled tension tourniquet was applied to the left limb in such a way that after surgical isolation through a short groin incision it spared the femoral neurovascular bundle. Thereby, subsequent arterial inflow was preserved for reperfusion. Complete arterial ischemia was achieved by selective clamping of the femoral artery whereas venous outflow was maintained during ischemia. Ischemia (4 hours) was followed by reperfusion for either 24 hours (n = 14) or 72 hours (n = 3). The above model characteristics were validated previously, as was the optimal timing of ischemia to achieve near lethal injury with a preserved therapeutic window. Analgesics (buprenorphin 50 μg/kg body weight) were administered half an hour before the end of each experiment and during postoperative follow up according to a pain monitoring protocol.

Muscle transfection

Somatic gene electrotransfer was used in the belly portion of the soleus and tibialis anterior muscles as described. In brief, plasmids were injected and their uptake was stimulated by electropulses. For tibialis anterior muscle 120 μl of plasmid (at 1 mg/ml in Gibco water for injection) was injected followed by eight trains of $60 \times 100$ μs pulses at 50 mA (interrupted by 994 ms) at three locations using needle electrodes on a GET42EV generator (GET42; EIP, Jonzieux, France). For soleus muscle 70 μl of plasmid was used followed by three trains of $80 \times 100$ μs pulses at 100 mA (interrupted by 992 ms) at two locations.

Muscle harvesting/handling

Muscles were rapidly excised under anesthesia and bluntly separated, frozen in melting isopentane, and stored at −80 °C in sealed cryotubes. Subsequently, 12 μm sections were produced for histochemical analyses. In parallel, cryosections were pooled for protein analyses. Transfection efficiency was verified in randomly selected pCMV-FAK-transfected specimens using immunohistochemical quantification of FAK protein content as described.

Endpoint analysis

Rhabdomyolysis and apoptosis were quantified on muscle cross sections after histological staining (rhabdomyolysis) or DNA fragmentation (apoptosis). The degree of inflammation was quantified using immunoblots for the macrophage marker CD68 and verified by the immunohistochemistry for CD68. The capacity to promote (BAX) or inhibit (Bcl-2) mitochondrial transition pores was assessed in immunoblots. Representative examples are shown in Fig. 2.

Rhabdomyolysis. Sections were Goldner stained, and proportions of intact versus lysed muscle fibers were quantified as described. In brief, microscopic fields (Eclipse TE2000-
In TUNEL positive nuclei were averaged per area measured. Immunohistochemistry for CD68. In PAGE, 20 μL Laemmli sample buffer and separated using 12% SDS–polyacrylamide gel electrophoresis (PAGE). Extract corresponding to 20 μg total protein was loaded per lane and blotted onto a nitrocellulose membrane and subjected to immunodetection with established antibodies (GE Life Science, Glattbrugg, Switzerland), CD68 (MCA341R, AbD Serotec, Düsseldorf, Germany), and horseradish peroxidase-coupled secondary antibodies (OxPhos complex kit, Molecular Probes, Invitrogen, Basel, Switzerland), n-terminally transfected animals (pCMV, plasmid) or transfection, which was completed successfully in all transfected animals (n = 13). The expected oxidative phenotypes were reconfirmed: slow-type soleus muscle contained more mitochondrial protein than tibialis anterior (+61 ± 13%, p = 0.03) or gastrocnemius medialis (+24 ± 21%, p = 0.08), respectively. Moreover, BAX protein levels generally correlated well with rhabdomyolysis (r = 0.63, p < 0.001).

Apoptosis. Signs of nicked DNA (a typical sign of apoptosis) were detected on cryosections with a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (in situ cell death detection kit, Roche Diagnostics AG, Rotkreuz, Switzerland). Subsequently, the number of TUNEL positive nuclei was quantified per cross-sectional area: microscopic fields were recorded at 10× magnification in a chessboard like manner avoiding field overlap. Only fields from central muscle sections were considered. Image J software was used to determine the number of TUNEL positive nuclei that demonstrated TUNEL (green) and nuclear stain (DAPI; blue) per microscopic field. Subsequently, TUNEL positive nuclei were averaged per area measured.

Inflammation. Infiltrating macrophages were detected by immunohistochemistry for CD68. Infiltration, like the capacity to modulate mitochondrial transition pores, was quantified using immunoblotting.

Immunoblotting. Total homogenates were denatured in Laemmli sample buffer and separated using 12% SDS–PAGE. Extract corresponding to 20 μg of total protein was loaded per lane reflecting the paired design of the experiment. Proteins were blotted onto a nitrocellulose membrane and subjected to immunodetection with established antibodies for FAK, Bcl-2, and BAX (#2870 and #2772, Cell Signaling Technology, Allschwil, Switzerland), mitochondrial proteins NDUFA9, SDHA, UQRC1, COX41, ARPSA1 (Anti-OxPhos complex kit, Molecular Probes, Invitrogen, Basel, Switzerland), CD68 (MCA341R, Abd Serotec, Düsseldorf, Germany), and horseradish peroxidase-coupled secondary antibodies (GE Life Science, Glattbrugg, Switzerland). Signal intensity was determined using a Femto chemiluminescent substrate (ThermoScientific, Reinach, Switzerland), recorded on X-ray film (GE Life Science, Glattbrugg, Switzerland) and scanned at 600 dpi into pdf format. Signal intensity was determined using Image J. To assess relative concentrations, each immunoblot was compared with a common sample.

Statistics

Sample sizes were determined based on expected effect sizes estimated from pilot experiments and minimized according to the “reduce—replace—refine” (3R) principle. Local effects were assessed using multifactor-analysis of variance (MANOVA) and Fisher post hoc tests for the factors “ischemic limb” (yes, no), “muscle type” (tibialis anterior, gastrocnemius medialis and soleus muscle), “ischemia reperfusion” (yes, no), and “plasmid” (pCMV, pCMV-FAK0 as appropriate (Statistica 10, Statsoft, Tulsa, USA). Systemic effects were assessed using MANOVA for the factors “ischemia” (yes, no), “ischemic muscle” (yes, no), and “muscle type”. Effects were assumed statistically significant at p < 0.05. Line graphs (rather than bars) were used to visualize effects or differences between conditions. Correlation analyses were carried out using Pearson’s coefficient.

RESULTS

Four of 29 rats (14%) died during the initial ischemic/procedural insult, but none died during subsequent reperfusion or transfection, which was completed successfully in all transfected animals (n = 13). The expected oxidative phenotypes were reconfirmed: slow-type soleus muscle contained more mitochondrial protein than tibialis anterior (+61 ± 13%, p = 0.03) or gastrocnemius medialis (+24 ± 21%, p = 0.08), respectively. Moreover, BAX protein levels generally correlated well with rhabdomyolysis (r = 0.63, p < 0.001).
Ischemia reperfusion without FAK over expression

In fast-type gastrocnemius muscle, rhabdomyolysis was already marked after 24 hours following IR (12% vs. 0.7% in paired controls, \( p = 0.002; \) Fig. 3A and Supplementary Fig. S1A). This finding persisted at 72 hours after IR. In contrast, slow-type soleus muscle did not demonstrate rhabdomyolysis at 24 hours of reperfusion, but only after 72 hours (3.1% vs. 0.7%, \( p = 0.02 \)). Apoptosis, however, was elevated in soleus muscle after 24 hours of IR (Fig. 3B).

Interestingly, BAX was not increased at 24 hours of reperfusion, in either native fast-type (\( p = 0.44 \)) or native slow-type muscles (\( p = 0.77 \)), compared with paired non-ischemic controls (Supplementary Fig. S2).

Looking at systemic effects, no difference was noted regarding rhabdomyolysis, apoptosis, or BAX levels in non-ischemic muscles from experimental rats compared to sham-treated animals (Supplementary Fig. S1).

Intrinsic FAK expression after reperfusion

FAK concentration was increased fivefold 24 hours after IR in fast-type muscle compared with paired controls (\( p = 0.002; \) Fig. 3C). A similar (although not statistically significant) trend was found in slow-type muscle (\( p = 0.11 \)). There was an interaction between muscle type, ischemic limb, and ischemia reperfusion (\( p = 0.06 \)) for FAK protein levels, indicating that the effect of IR on intrinsic FAK expression depended on muscle type and on whether the muscle was subjected to ischemia.

Effects of enhanced FAK expression

pCMV-FAK transfection was effective in fast-type muscle, where both ischemic and non-ischemic limbs showed increased FAK concentrations (2.5-fold and 2.1-fold, respectively) compared with empty plasmid-transfected controls 24 hours after reperfusion (Fig. 4A). Consequently, IR-induced rhabdomyolysis did not increase over baseline level (i.e., over the level in the paired, non-ischemic controls) in pCMV-FAK transfected fast-type muscle (Fig. 4B). In contrast, empty plasmid-transfected muscle, similar to native muscle, demonstrated increased rhabdomyolysis compared to the paired, non-ischemic controls with IR (Fig. 4B). Consistent with these findings, IR-related BAX protein content was sixfold reduced following pCMV-FAK transfection (Fig. 4C), whereas Bcl-2 and apoptosis were not affected by pCMV-FAK transfection (Fig. 4D/E). Lastly, pCMV-FAK transfection produced a reduction in the concentration of macrophage marker CD68 in tibialis anterior muscle of the ischemic limb (\( p = 0.01 \)), which was elevated with empty transfection with IR (Fig. 4F).

In slow-type muscle, pCMV-FAK transfection did not increase FAK protein concentrations (\( p = 0.66 \), compared with empty plasmid controls), in either ischemic or non-ischemic muscle.
controls (Fig. 5A). Consistently, FAK transfection did not affect Bcl-2 (Fig. 5B), BAX (\( p = 0.35 \)) or CD68 protein levels (\( p = 0.69 \)) after IR (data not shown). However, pCMV-FAK transfection reduced apoptosis with IR (Fig. 5D). Transfection with empty plasmid produced rhabdomyolysis in ischemic and non-ischemic slow-type muscle to a similar extent (13.7% and 12.3%, respectively, \( p = 0.22 \)) after 24 hours of IR.

**DISCUSSION**

IR injury is a fundamental and universal pathogenic process. Unsurprisingly, it has attracted unparalleled attention within cardiovascular research, \( ^{3} \) which so far has focused mainly on cardiac IR injury. \( ^{5,21,22} \) As a consequence, management of acute myocardial infarction has been revolutionized over the years. \( ^{8,9} \) Based on fundamental differences, these insights may or may not be applicable to the clinical management of acute limb ischemia. FAK’s protective effects on acutely ischemic cardiac muscle \( ^{7,23} \) primed the present hypothesis driven investigation, which reconfirmed a similar effect in fast-type skeletal muscle. Therefore, the present pilot findings may provide important impetus for research into practical salvage strategies that may be effective at least in a portion of the musculature at risk.

FAK is believed to convey a strong survival signal based on the following observations: only rapid degradation of FAK allows for apoptosis during ischemia \( ^{7,8} \); FAK reacts with a rebound increase upon reperfusion after ischemia \( ^{13} \); and this reaction may be maximized by repeated brief bouts of ischemia (so called “pre-conditioning”), which seems highly effective in protecting cardiac muscle from IR injury. \( ^{7,9,24} \)

The main findings of the present investigation support a beneficial effect of FAK in ischemic skeletal muscle, modulated by the oxidative muscle phenotype (i.e., mitochondria content). Using electro-assisted gene transfer, a muscle type-specific disposition to express FAK was identified, as well as differential effects of FAK on rhabdomyolysis and modulators of mitochondrial transition pores (i.e., BAX, Bcl-2). These protective biological effects appeared only in fast-type muscle but not in slow-type muscle, which did not respond to transfer with pCMV-FAK. Intriguingly, slow-type muscle appeared naturally better protected against IR injury than fast-type muscle. A manifest interpretation is that mitochondria-poor skeletal muscle cells may benefit more from increased FAK levels because their functional reserves for mitochondrial biogenesis are larger. In mice, for instance, fast-type muscle fibers present a lower mitochondrial content than slow-type muscle and appear

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**Figure 5.** Ischemia reperfusion injury in slow-type muscle (soleus muscle) with FAK over expression. Bar graphs visualizing the mean \( \pm \) SD of FAK (A) and Bcl-2 (B) protein levels, the mean related percentage of rhabdomyolysis (C), fold changes in apoptosis (D) in transfected soleus muscle of the ischemic and non-ischemic limb. \( +0.05 < p \leq 0.10 \) vs. pCMV (ANOVA with the Fisher post hoc test).

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relatively protected by FAK, which is in line with the postulated prevention of apoptosis by FAK. They find that FAK reduces the expression of the transition pore promoter BAX while enhancing mitochondrial biogenesis via increased Bcl-2 levels. FAK signaling has been described previously to stabilize mitochondria in culture by up-regulating Bcl-2, and to counteract the adverse effects of BAX, that is mitochondrial transition pore opening and apoptosis. This effect was paralleled by a reduced number of lysed muscle fibers and the inflammation marker CD68, which are known to increase with mitochondrial calcium overload after IR injury when mitochondrial functioning is inhibited (Fig. 1A). These combined effects make FAK a promising candidate for skeletal muscle IR management, at least in fast-type muscle.

In contrast, in muscle cells that are already rich in mitochondria their upregulation may not have a corresponding effect. In the present study, the relative inefficacy of FAK in slow-type muscle suggests that the prevention of IR-induced lysis and apoptosis is graded by pre-existing mitochondrial content, which could explain the observed ceiling effect of FAK expression levels.

The severity of inflammation and rhabdomyolysis was modulated consensually with FAK effects on apoptosis. CD68-positive macrophages remove debris of damaged fibers thereby reducing escalation of inflammation. FAK overexpression is known to enhance transcript levels of immune cell markers. The findings of a reduction in BAX and CD68 content and rhabdomyolysis indicate that FAK overexpression lowers the degree of IR damage in tibialis anterior muscle by affecting the inflammatory response.

Taken together, the present findings highlight the importance of sustained (FAK-regulated) mitochondrial integrity for salvage of ischemic skeletal muscle, which depends on the oxidative phenotype. This may be exploited therapeutically. For instance, an antibody therapy targeted at beta-1-integrin, an upstream regulator of FAK phosphorylation, has been shown to protect tubular epithelia against kidney IR injury by preserving their adhesive cell state. And FAK overexpression is known to increase the concentration of b1 integrin-based focal adhesions in muscle fibers, suggesting an important role in the stabilization of muscle cell membranes.

Strengths and limitations

A translational strength of the study is the realistic emulation of arterial limb ischemia. Obstruction of the venous outflow (and thus halted cellular respiration) is a typical confounder of previous small animal (tourniquet) models of limb ischemia, as is damage to the arterial inflow preventing reliable in vivo assessment of reperfusion injury or treatment effects. The present experiments circumvented these limitations. Moreover, this model theoretically allows intra-arterial application of therapeutic agents during ischemia without affecting the femoral artery (i.e., via cannulation of the epigastric artery). This could be relevant for future experiments.

The most important limitation is that FAK expression was enhanced before onset of ischemia, which was due to the proof of concept approach. However, in the clinical reality of acute limb ischemia, post-conditioning approaches that are effective in established ischemia are obviously needed. It is known from cardiac research that therapeutic RISK activation protects ischemic cardiac muscle as long as it occurs timely before reperfusion. Pharmacological measures targeted at proximal integrin signaling as outlined above may be of potential use for post-ischemia conditioning of skeletal muscle and should be investigated in future research. However, preconditioning is theoretically feasible before iatrogenic ischemia, for instance during orthopedic arthroplasty (arterial tourniquet) or peripheral revascularization, and might help in the mitigation of collateral IR injury. Therefore, the present findings retain by all means a concrete clinical relevance. Future research must aim at assessing whether the damage response to IR is also graded by mitochondrial reserves and FAK protein levels in humans and whether the aerobic phenotype of muscles being affected by IR may help refining priorities during surgical interventions to prevent irreversible muscle damage.

Another limitation is that experiments were designed to assess the impact of FAK on cellular IR injury endpoints rather than on underlying signaling pathways. Thereby, contralateral limbs were used as controls for inflammatory markers. Although these muscles showed no difference to sham treated controls, IR could have affected them by systemic effects, thereby confounding the postulated FAK-mediated protection. But even if that was the case, the true difference between ischemic and non-ischemic muscle would have been underestimated, which strengthens the conclusions. All findings, albeit in line with the study hypothesis and previous experimental evidence, are based on modest numbers of biological replicas, which might affect statistical power. As the experiments were set up as a pilot study to evaluate salvage pathways known to be effective in myocardial infarction, they aimed to comply with the demands of the 3R principle. To control the multiple biological factors, assessed time points were limited according to established IR injury kinetics in this model. Last but not least, potentially important factors such as intracellular calcium metabolism were not controlled, which could confound any conclusions. Despite these limitations, the experiments produced consistent, plausible, and statistically significant results suggesting that the extent of skeletal muscle IR injury was modified by muscle phenotype and could be mitigated by enhanced FAK expression. Arguably, biological effects after IR seem particularly complex in peripheral muscle and the investigated therapeutic approach was not effective in all muscle types.

In conclusion, the present findings suggest a modulatory role of the oxidative muscle phenotype in IR-induced rhabdomyolysis, which is likely to correlate with muscle fiber mitochondria content. The identified protective effect of enhanced FAK expression is fast-type muscle specific and offers a potential therapeutic approach to reduce reperfusion induced rhabdomyolysis preserving muscle function.
For clinical benefit, appropriate strategies must be identified that are effective while ischemia is already established and could be included in post conditioning protocols before conclusions regarding translational potential and clinical relevance can be drawn.

CONFLICT OF INTEREST
None.

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APPENDIX A. SUPPLEMENTARY DATA
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejvs.2014.11.011

REFERENCES


