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Poly ADP-Ribose Polymerase Inhibition Ameliorates Hind Limb Ischemia Reperfusion Injury in a Murine Model of Type 2 Diabetes

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Abstract

Introduction—Diabetes is known to increase poly-ADP-ribose-polymerase (PARP) activity and posttranslational poly-ADP-ribosylation of several regulatory proteins involved in inflammation and energy metabolism. These experiments test the hypothesis that PARP inhibition will modulate hind limb ischemia reperfusion (IR) in a mouse model of type-II diabetes; ameliorate the ribosylation and the activity/transnuclear localization of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Methods—db/db mice underwent 1.5hrs of hind limb ischemia followed by 1, 7, or 24hrs reperfusion. The treatment group received the PARP inhibitor PJ34 (PJ34) over a 24hrs period; the untreated group received Lactated ringer's (LR) at the same time points. IR muscles were analyzed for indices of PARP activity, fiber injury, metabolic activity, inflammation, GAPDH activity /intracellular localization and poly-ADP-ribosylation of GAPDH.

Results—PARP activity was significantly lower in the PJ34 treated groups compared to the LR group at 7 and 24 hours reperfusion. There was significantly less muscle fiber injury in the PJ34 treated group compared to LR treated mice at 24 hrs reperfusion. PJ34 lowered levels of select proinflammatory molecules at 7hrs and 24hrs IR. There were significant increases in metabolic activity only at 24 hours IR in the PJ34 group, which temporally correlated with increase in GAPDH activity, decreased GAPDH poly ADP-ribosylation and nuclear translocation of GAPDH.

Conclusions—PJ34 reduced PARP activity, GAPDH ribosylation, GAPDH translocation, ameliorated muscle fiber injury, and increased metabolic activity following hind limb IR injury in

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a murine model of type-II diabetes. PARP inhibition might be a therapeutic strategy following IR in diabetic humans.

INTRODUCTION

Type II Diabetes Mellitus (DM2) affects more than 23 million people in the United States, representing roughly 7–8% of the total population^{1, 2}. DM2 is a common co-morbidity encountered with peripheral arterial disease (PAD), and together account for the majority of non-traumatic amputations among the diabetic population. Studies have demonstrated that in human diabetic tissues there is an increase in oxidative, nitrosative stresses and genomic instability, leading to an up-regulation in poly ADP-Ribose polymerase activity (PARP)^{3–6}. PARP is a nuclear and mitochondrial enzyme involved in numerous cellular activities, including DNA repair, maintenance of genomic integrity, modulation of various proteins at the transcriptional and post-transcriptional levels, regulation of cell death, cellular replication and differentiation^{7, 8}. Under pathologic conditions however, extensive PARP activation in response to stress can lead to NAD⁺ depletion, which compromises glycolysis and ATP generation^{5, 9}. Increased PARP activity is believed to play a critical role in the etiology of vascular injury and complications seen in DM2 patients by decreasing vasomotor reactivity in skin and systemic arteries^{6, 10, 11}.

Recent investigations have focused on the ability of PARP to modify enzymatic activity by covalent ribosylation^{7, 8}. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate. It has been reported that PARP activation modulates the activity of GAPDH by post translational poly-ADP-ribosylation in hyperglycemic stress (in vitro) and renal tissue subjected to global ischemia reperfusion in vivo^{5, 12, 13}. Furthermore, there is literature to suggest that the GAPDH, normally a cytoplasmic enzyme, can undergo nuclear translocation during conditions of ischemia (retinal) and ischemia reperfusion (brain) where its expression correlates directly with the severity of tissue injury^{14, 15}. It is not known whether GAPDH undergoes ribosylation and/or nuclear translocation in the setting of hind limb ischemia reperfusion. Previous reports from our lab demonstrated that PARP inhibition preserves energy substrates, mitochondrial activity and skeletal muscle fiber integrity in normal wild type mice subjected to acute limb ischemia-reperfusion injury (IR)^{16–19}. To date, the effect of PARP inhibition on hind limb IR in an *in vivo* model of type II diabetes has not been investigated. Since IR is considered to be a major component in the development of decubitus ulcers, wound healing, peripheral vascular disease, and transplantation in diabetic patients^{20–22}, these experiments were designed to determine whether PARP inhibition modulates tissue injury, inflammation, indices of metabolic function and GAPDH expression/activity and intracellular localization in a murine model of DM2.

METHODS

Animal protocol

All experimental procedures were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in accordance with the “Principles of the Laboratory Animal Care” (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication NO. 86-23, Revised 1996). 10–12 week-old male Leptin receptor deficient strain B6. BKS (D) Lepr^{db} mice were acquired from the Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized via intraperitoneal administration of pentobarbital 50–60mg/kg in normal saline. IR mice underwent a period 1.5 hours of unilateral hind limb ischemia followed by 24 hrs reperfusion which was confirmed using laser doppler imaging (Moor Instruments Inc., Wilmington, DE) as previously described²³.

Mice remained anesthetized throughout the duration of ischemia and were kept on a regulated warming plate to ensure normothermic core body temperature.

Treatment Protocol

To evaluate skeletal muscle responses to ischemia reperfusion, mice were divided into three major groups. The untreated group (LR, $n = 27$) received intraperitoneal administration of 0.5ml plain Lactated Ringer's solution at 5 minutes prior to reperfusion, and at 15 minutes, 2 hours (3 dose LR therapy), or an additional 6 hours, 12 hours, and 18 hours reperfusion (6 dose LR therapy). The PARP inhibitor treatment group ($n = 30$) received intraperitoneal injections of 30 mg/kg doses of PJ34 dissolved in 0.5ml LR at either 5 minutes prior to reperfusion, 15 minutes, or 2 hours reperfusion (3 dose PJ34 therapy) or for an additional 6 hours, 12 hours and 18 hours reperfusion (total 6 dose PJ34 therapy) at the same time periods. Subgroups of mice from each group were euthanized at 1 ($n = 6-8$), 7 ($n = 7-8$) or 24 hours ($n = 14$) reperfusion. Mice in the sham group ($n = 8$) were subjected to anesthesia alone for 1.5 hours, followed by a 24 hour period of recovery were used as control. The IR and contralateral hind limbs were harvested for analytical evaluation. The posterior calf muscles were excised and immediately frozen in liquid nitrogen for biochemical or molecular analysis, while the anterior tibialis muscles were fixed and processed for histological evaluation. PJ34, a novel, potent phenanthridinone derivative PARP inhibitor was chosen for these studies because it is water soluble and effective in ameliorating a variety of inflammatory processes in murine and rodent models^{24, 25}. The precise half life for PJ34 in mice is not known, however in rats it has been measured as approximately two hours²⁶.

Histologic Evaluation

The hind limb muscles (anterior compartment) were fixed in 4% paraformaldehyde overnight, then rinsed in phosphate buffered saline for 1 hour, followed by serial dehydration in graded acetone. The muscle samples were embedded in the acrylic compound JB-4 kit (Electron Microscopy Sciences, Hatfield, PA). Quantitative microscopic evaluation of the anterior tibialis muscle was performed to obtain the percentage of injured muscle. The muscle fibers were counted and scored (as uninjured or injured) based on the protocol and morphology as previously reported²⁷.

Metabolic Assessment

200mg hind limb frozen muscle samples were homogenized in 10% Trichloroacetic acid and analyzed for the steady state levels of ATP & L-lactate (measures of metabolic activity) using chemiluminescence and spectrophotometric assays, respectively, as previously described²⁸.

Western blotting analysis for PARP activity, Total GAPDH protein

Frozen skeletal muscle tissues were homogenized on ice with lysis buffer containing 20 mM Tris, pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM sodium pyrophosphate, 2mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate and mammalian cells protease inhibitor cocktail (Sigma-Aldrich, St Louise MO). Tissue lysates were centrifuged at 10000xg at 4°C for 10 min, then aliquoted and kept frozen until further analysis. PARP activity was assessed by western blotting for the solubilized poly ADP-ribosylated proteins (PAR) using monoclonal anti-poly-(ADP)-ribose moiety antibody at 1: 2000 (Tulip Biolabs, West Point, PA) as previously described¹⁸.

The expression of the 37kDa GAPDH protein was detected in aliquots of 50µl of total protein extracts by western blotting using 2ug/ml sheep anti-GAPDH polyclonal antibody (Novus Biologicals, Littleton, CO) and Donkey anti-sheep HRP conjugated IgG at 1:10000 dilution. The generated specific protein bands densities were quantified using FluorChem HD2 Imaging system software (Cell Biosciences, Santa Clara, CA). The blots were stained with Ponceau S staining and imaged to demonstrate evidence of equal protein loading into each lane. The 42kDa band densities were used to normalize the specific bands density values. Data was expressed as percent sham values at each time point.

PAR Immunohistochemistry

Deparaffinized 7µm sections were permeabilized with 0.1% Triton x-100 then subjected to nonspecific blocking using Avidin and Biotin blocking (Gibco life technologies) and mouse tissue on mouse protein blocking reagent (M.O.M. blocking kit, Vector Laboratories, CA) for 60 minutes at room temperature then incubated with 1:100 dilution of mouse anti PAR monoclonal IgG (Tulip Biolabs) at 4°C followed by incubation with horse anti mouse biotinylated IgG. The section were hybridized and developed with horseradish peroxidase conjugated Streptavidin for 30 minutes and 3,3 Diaminobenzidine (DAB) respectively (Gibco life technologies). Images were acquired with light microscopy and Spot Insight digital camera.

Local Markers of Inflammation

Selected markers of inflammation were detected from muscle extracts using quantitative sandwich enzyme immunoassays for Keratinocyte Chemoattractant Protein (KC), interleukin-6 (IL-6) (R&D Systems, Minneapolis, MN) and myeloperoxidase (mouse MPO, Cell Sciences, Canton, MA). The ELISA plates were read with Spectromax-250 plate reader (Molecular Devices, Sunnyvale, CA). The values were extrapolated from the standard curve and normalized to the total protein concentration, which was determined with the Bicinchoninic Acid (BCA) Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL)²⁹.

GAPDH Poly ADP-Ribosylation Detection

To assess ribosylation of GAPDH, 1000ug of total protein from tissue lysates was immunoprecipitated with 25ug/ml anti-poly ADP-ribose moieties polyclonal IgG (Enzo Life Sciences, Farmingdale, NY). Protein A/G conjugated agarose beads immobilized inside a column (Pierce Classic IP Kit, Thermo Scientific, Waltham, MA) were used to collect the Poly ADP Ribosylated proteins. The eluted proteins were subjected to western blotting detection using 12% polyacrylamide gel electrophoresis and transfer to a nitrocellulose membrane. Proteins were hybridized with 2ug/ml sheep anti-GAPDH polyclonal IgG (Novus Biologicals, Littleton, CO), followed by incubation with donkey anti-sheep horseradish peroxidase conjugated secondary IgG at 1:10,000 dilution (Novus Biologicals, Littleton, CO). Protein bands were detected with ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ). Bands were visualized with FluorChem HD2 imaging system (ProteinSimple, Santa Clara, CA), and bands densities were quantified using the imaging system software.

Measurement of GAPDH Activity

GAPDH activity was evaluated using a kit (Biomedical Research Service Center, University of Buffalo, Buffalo, NY). The kit is based on the enzymatic reduction of the tetrazolium salt in a NADH coupled enzymatic reaction to form formazan. In brief, 100ug frozen muscle tissues were homogenized in ice-cold lysis buffer. Samples were cleared by centrifugation, and the total protein concentration was measured in the supernatant with BCA protein assay.

2mg of total protein aliquots were reacted with the reagent mix in a 96 multi-well plate according to the manufacturer's instructions. Purified rabbit muscle GAPDH enzyme (G2267, Sigma-Aldrich) was used to generate a linear standard curve for comparison to the experimental samples harvested from mice. The reacted plates were read with Spectromax-250 plate reader (Molecular Devices, Sunnyvale, CA). The values were extrapolated from the standard curve values and expressed as Units/2 mg of total protein.

GAPDH and PAR Colocalization

Deparaffinized 7µm sections were permeabilized with 0.1% Triton x-100 then subjected to nonspecific protein blocking with MOM reagent (Vector Laboratories) for 60 minutes then hybridized at room temperature with mouse anti PAR monoclonal IgG (Tulip) at 1:100 dilution overnight at 4C followed by incubation with rabbit anti GAPDH polyclonal IgG (AbCam) at 1:1000 dilution for 1 hour at room temperature. immunofluorescence detection was carried out by incubating the slides sequentially with goat anti-mouse Cy3 conjugated IgG then goat anti-rabbit FITC conjugated IgG (GIBCO Life Sciences) for 1 hour at room temperature. Slides were mounted with vectashield anti fading hard set mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) DNA stain (Vector Laboratories, Burlingame CA). Slides were imaged using Olympus BX51 fluorescent microscope and Nuance multispectral imaging system with corresponding filter sets. Images were colored and processed using ImageJ software (National Institute of Health, imgj.nih.gov).

Statistical Analysis

Analysis within the group were done by one-way ANOVA with Tukey post hoc analysis and between the two group by unpaired student t-test using GraphPad Instat3 software.

RESULTS

Assessment of Skeletal Muscle Fiber Injury

Histologic evaluation of skeletal muscle fiber morphology at 24 hours IR after 3 doses of PJ34 showed no difference in muscle fiber injury as compared to LR treated mice ($p > 0.05$). In contrast, mice treated with 6 doses of PJ34 had preservation of fiber morphology as compared to the LR and sham groups (Figure 1, top). The LR group demonstrated large areas of highly injured muscle fibers characterized by loss of the polygonal cellular shape, loss of tight cell adhesion, cytoplasmic irregularities and cell wall/membrane disruption. Quantitative assessment indicated markedly lower percentage of fiber injury in the PJ34 treated groups compared to the LR group (PJ34: 12.9 ± 2.1 ; vs. LR: 20 ± 4.4 percent, $p = 0.0008$, Figure 1, bottom).

Temporal skeletal muscle PARP activity following IR

Immunoblotting showed evidence of substantial PARP activity in IR mice as compared to sham mice (representative immunoblot in Figure 2). PARP activity similarly increased in both treated and untreated groups following one hour IR (LR: 191.62 ± 18.96 ; PJ34: 199.92 ± 20.55 percent, $P < 0.05$, Figure 3A). However, PJ34 treatment significantly reduced PARP activity by 7 and 24 hours reperfusion (at 7 hrs; LR: 245.05 ± 19.1 vs. PJ34: 160.16 ± 14.71 percent sham, $p = 0.009$ and at 24hrs; LR 315.48 ± 42.96 vs. PJ34 220.96 ± 20.42 percent sham, $p = 0.004$, Figure 3A) compared to the mice treated with LR. PJ34 treatment did not alter the relative expression of the major PARP-1 isoform at 24 hours reperfusion (LR: 131 ± 30 , PJ34: 118 ± 19 percent sham, $p = 0.7$, Figure 3B).

Histologic Localization of PARP Activity

Immunostaining of PAR in hind limb skeletal muscle tissue exhibited increased detection of PARP activity localized to the peripheral nuclei of the skeletal muscle fibers in LR and PJ34 treated mice subjected to IR (Figure 4, solid black arrows panels C and D) compared to sham (panel B). In addition, in LR treated mice, there is evidence of PAR activity in intravascular and interstitial inflammatory cells (dotted arrows panel C and D). There was no evidence of positive immunostaining for PAR in the absence of the primary antibody (panel A).

Markers of Inflammation

There was no significant difference in the local skeletal muscle protein levels of any markers of inflammation after 1 hour reperfusion (Table 1). However, after 7 hours reperfusion KC and MPO levels were significantly lower in the PJ34 treated group compared to LR. Skeletal muscle MPO levels increased significantly after 24 hours IR compared to 1 hour IR in LR and PJ34 treated mice ($p < 0.001$). However, there was no significant difference observed between the treated and untreated groups in the measured pro-inflammatory acute phase reactants, KC, and MPO at 24 hours. In contrast, IL-6 protein levels were similar between the PJ34 treated and LR treated groups at 1, 7 and 24 hour intervals.

Markers of Metabolic Activity

Skeletal muscle L-lactate levels in the LR group were significantly higher after 1 hour reperfusion compared to PJ34 treated mice (Table 2). Lactate levels in the LR group were slightly higher at 7 hours reperfusion but this did not reach statistical significance. By 24 hours reperfusion, the PJ34 treated group had significantly higher level of lactate compared to the LR group. Skeletal muscle ATP levels were identical after 1 and 7 hour IR in the LR and PJ34 treated groups (Table 2). By 24 hours IR, the PJ34 treated mice did have significantly higher levels of ATP compared to LR treated mice.

Skeletal muscle GAPDH protein expression and activity

To determine whether the metabolic rescue effect of PJ34 on skeletal muscle tissue metabolic activity (i.e. increased lactate and tissue ATP) was associated with alterations in GAPDH protein expression, immunoblotting of skeletal muscle protein extracts was performed from samples harvested at 1, 7 and 24 hours reperfusion (Figure 5A). There was no significant difference in relative GAPDH protein expression at 1 or 7 hours reperfusion in PJ34 treated vs LR treated mice. However, by 24 hours reperfusion there was a slightly lower relative GAPDH protein expression in the PJ34 treated group (Figure 5B). Despite the findings of decreased overall GAPDH protein expression at 24 hours reperfusion, GAPDH activity was significantly higher in the hind limbs skeletal muscles from the PJ34 treated group compared to LR at the same time period (LR, 38.89 ± 4.53 vs. PJ34, 62.01 ± 5.15 , $p = 0.005$, Figure 6).

GAPDH protein Poly ADP-Ribosylation

To explain the discrepancy between the absolute expression of GAPDH protein and GAPDH activity after treatment with PJ34, an analysis of GAPDH ribosylation was performed. Ribosylation of GAPDH is known to be associated with decreased GAPDH activity^{12, 13}. Western blot detection of immunoprecipitated poly ADP-ribosylated protein after 24 hours IR (Figure 7A) in the hind limb skeletal muscle tissues lysate from sham, LR treated, and PJ34 treated mice indicate that GAPDH poly ADP-ribosylation is significantly enhanced during reperfusion in the LR group compared to sham (Sham: 0.49 ± 0.06 vs LR: 1.5 ± 0.22 , $p < 0.05$, Figure 7B). In contrast PJ34 treatment markedly reduced the poly

ADP-ribosylation of GAPDH at 24 hours reperfusion (LR 1.5 ± 0.22 vs PJ34: 0.41 ± 0.13 , $p < 0.01$, Figure 7B).

PAR Activity and GAPDH Subcellular localization

Since nuclear translocation of GAPDH is known to be associated with ischemic stresses^{14, 15}, dual immunofluorescence staining on hindlimb TA muscle was employed to determine the cellular localization of GAPDH enzyme and PAR activity under non ischemic conditions (i.e. sham) and following ischemia reperfusion with and without PJ34 (Figure 8). In the sham group, GAPDH was diffusely present in the cytoplasm of the muscle fibers (green hue); There was also a very low level of PAR activity (red speckles) under sham conditions. Under sham conditions, there was little evidence of significant colocalization of GAPDH and PAR activity (green GAPDH enzyme plus red PAR activity = orange colocalized GAPDH and PAR). In contrast, reperfusion injury resulted in enhanced nuclear PAR activity (red speckles, panel E, Figure 8) and nuclear translocation of GAPDH with increased PAR detection in nuclei (orange arrows and speckles in panel F). Finally, PJ34 treatment dramatically reduced PAR activity and partially ameliorated nuclear colocalization of PAR and GAPDH at 24 hours IR (Figure 8).

DISCUSSION

These experiments demonstrate that systemic administration of the PARP inhibitor PJ34 during acute ischemia reperfusion injury in db/db mice decreased tissue injury as assessed by histologic and biochemical criteria (Figure 1, Table 2). This PJ34 mediated decrease in tissue injury was associated with substantial preservation of GAPDH activity, decreased PARP activity, ribosylation of GAPDH and reduction in nuclear translocation of GAPDH. Ischemia-Reperfusion is a major component of tissue injury in diabetic humans^{3, 30–33}. The db/db mouse was selected for studies of a murine model of human type 2 diabetes because the mice are phenotypically obese, and are known to be hyperglycemic and hypercholesterolemic¹⁸.

For these experiments, a clinically relevant post hoc treatment protocol was utilized²⁸. Since it is difficult to predict the precise circumstances when an ischemic insult might develop, the administration of a treatment protocol after the onset of the injury seemed to be the most clinically relevant scenario. Since these experiments employed a previously defined treatment protocol in a different mouse strain, experiments were performed to confirm that PAR activity was altered by the administration of the inhibitor. Post hoc administration of PJ34 significantly decreased the activity of PARP enzymes (Figure 3A). Administration of PJ34 clearly altered PARP activity, but did not alter the steady state levels of PARP-1 enzyme in skeletal muscle at 24 hours after ischemia reperfusion (Figure 3B).

Immunohistochemical analysis of the localization of PARP activity was performed to determine the cellular localization of PARP activity in skeletal muscle (Figure 4). During sham conditions showed minimal signal (Figure 4, panel B). In contrast, IR in skeletal muscle of untreated mice showed evidence of PARP activity in the peripheral nuclei and intravascular spaces (Figure 4C). It is likely that PARP activity in the intravascular spaces may represent activity in inflammatory cells. PJ34 treatment markedly decreased expression of PARP activity in both muscle nuclei and the intravascular spaces (Figure 4D). This finding is consistent with the concept that PARP inhibition can modulate intracellular and extracellular sources of oxidative stress during ischemia reperfusion.

In contrast to previous reports from our laboratory on the effect of PJ34 treatment in wild type mice^{17, 34}, administration of this drug to db/db mice did not reduce all the levels of markers of skeletal muscle inflammation throughout the entire period of reperfusion (Table

1). Experimental studies of cardiac and renal IR in the setting of DM2 have demonstrated a more robust inflammatory response following injury^{35–37}. It is also possible that epigenetic mechanisms known to modulate chronic conditions associated with diabetes, might alter the tissue response to PARP inhibition^{38, 39}. KC is the murine analogue for human IL-8, a potent neutrophil chemoattractant and activating protein which has been implicated as contributing to tissue injury in a number of animal models of tissue ischemia reperfusion^{40, 41}. An analysis of this murine equivalent of human Interleukin-8 (IL-8) is relevant because IL-8 is known to be elevated in the serum of human claudicants⁴². Despite the absence of decreased KC at 24 hours reperfusion, there was a significant decrease in MPO expression at this time point in the PJ34 treated mice. Since MPO activity is believed to be a major contributor to leukocyte mediated tissue cytotoxicity, these findings suggest that cytokines other than KC are likely responsible for the decrease in skeletal muscle MPO and tissue injury at 24 hours reperfusion in diabetic mice. Interleukin-6 levels were not different in LR vs PJ34 treated mice at any experimental interval. Interleukin-6 has been found to be elevated in the plasma of humans⁴³ and mice¹⁶ subjected to acute hind limb tourniquet ischemia. IL-6 has also been shown to be upregulated in patients with PAD, claudication^{44–46}, and in those undergoing vascular bypass procedures⁴⁷. There is literature to suggest that IL-6 can provide cytoprotection against various forms of acute tissue injury^{48–50}, thus the finding that PJ34 did not reduce skeletal muscle levels of this cytokine might not be a sign of a failed therapeutic intervention.

PJ34 administration significantly increased ATP and L-Lactate levels at 24 hours reperfusion (Table 2). L-lactate is the end product of anaerobic metabolism, thus its increase in skeletal muscle likely represents the greater number of viable cells able to undergo anaerobic metabolism (i.e. glycolysis). The increase in ATP levels illustrates a relative preservation of energy stores needed for cell homeostasis. Recent studies have shown that GAPDH, known to be a key glycolytic enzyme is also a protein with multiple cytoplasmic, membrane, and nuclear functions and is a major intracellular messenger mediating apoptosis of cells. GAPDH translocation to the nucleus is considered an important step in glucose-induced apoptosis of retinal Muller cells¹⁴. The mechanism that initiates its translocation is not well understood, however covalent modification by nitration/ribosylation is considered the most likely possibility^{13, 15}. When GAPDH is ribosylated, its enzymatic activity is functionally inhibited, resulting in an inhibition of glycolysis and decreased tissue levels of ATP and lactate¹². Since both lactate and ATP levels were relatively preserved by treatment with PJ34, a quantitative analysis of the overall expression of GAPDH protein, tissue expression of ribosylated GAPDH or GAPDH activity was undertaken. Our initial assessment of total GAPDH protein expression revealed that PJ34 treated mice had less GAPDH protein than their LR treated counterparts at the 24 hour period (Figure 5A). This was an unexpected finding since GAPDH is a constitutively expressed protein, however there is data to suggest that GAPDH is a preferential target for oxidative stress. Oxidized LDL and hydrogen peroxides, known mediators of oxidative stress have been shown to decrease GAPDH protein stability and therefore GAPDH protein expression⁵¹. Evidence to support the concept that the observed reduction in total GAPDH protein expression did not represent a physiologic compromise of skeletal muscle was provided by assessment of GAPDH activity. Despite the decrease in GAPDH protein expression, GAPDH activity in the PJ34 treated mice was significantly greater than the LR treated mice (Figure 6). This increase in GAPDH activity associated with the administration of PJ34 did also correlate with decreased expression of ribosylated GAPDH (Figure 7A & B). This finding is quantitatively and qualitatively consistent with the findings regarding GAPDH expression in a murine model of renal ischemia¹².

In addition to its crucial role in glycolysis, there is also considerable evidence to support a role of GAPDH in nuclear transcription. Studies of skeletal muscle hypoxia⁵² and stress⁵³ in

tissue culture models support a role for GAPDH in nuclear transcription. Nuclear translocation of GAPDH has been shown to directly correlate with the extent of tissue injury in ischemic and reperfused tissue^{14, 15}. To determine whether there was evidence of nuclear translocation of GAPDH during ischemia and its relationship to ribosylation, immunofluorescent studies were undertaken (Figure 8). Under sham conditions, GAPDH expression (green FITC) was diffusely localized primarily in the periphery and cytoplasm of the skeletal muscle fibers. In contrast PAR expression (red, Cy3) can be barely visualized in the peripheral skeletal muscle nuclei. This is an expected finding under sham conditions as is the finding of minimal colocalization of PAR and GAPDH in the nucleus (Panel C). This histologic finding is consistent with our immunoblotting results which showed minimal PAR activity (Figure 2) and ribosylated GAPDH expression (Figure 5A) under sham conditions. In contrast, during ischemia reperfusion in the LR treated mice, there is substantial evidence of PAR and GAPDH colocalization in the punctuate nuclei of the skeletal muscle as demonstrated by the large number orange speckled areas in the periphery of the muscle fibers (panel F). PJ34 treatment substantially reduced degree of PAR-GAPDH colocalization in the nucleus of reperfused skeletal muscle fibers (panel I). This is the first *in vivo* demonstration of colocalization of PAR and GAPDH in any tissue during reperfusion injury, and its reversal by a PARP inhibitor.

In conclusion, PARP inhibition is an effective treatment strategy in managing acute hind limb ischemia reperfusion in the diabetic mouse. Ongoing basic and clinical evaluation of the role of PARP inhibitors in the management of human clinical problems is justified since clinical trials of this class of drugs in the management of breast cancer, solid and lymphoid tumors has been encouraging. Ongoing mechanistic assessment of how PARP inhibitors alter cellular physiology are necessary to understand what conditions might be relevant for further clinical investigations. To that end, these studies are the first to document alteration of GAPDH function associated with its degree of ribosylation in skeletal muscle following ischemia reperfusion, along with *in vivo* colocalization of PAR and GAPDH in the nucleus following an reperfusion injury. Further investigation focusing on tissue healing and functional recovery using an extended interval of PARP inhibition protocols are warranted to better assess the potential clinical application of this experimental therapy.

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Figure 1.

(top). Representative photomicrographs of skeletal muscle fibers cross sections. (A) Normal skeletal muscle tissue with polygonal cellular architecture, peripherally located nuclei, and tight fiber pattern. (B) IR skeletal muscle from the LR treatment group at 24 hours reperfusion, demonstrating significant fiber necrosis with complete loss of the polygonal architecture, nuclear staining, fiber wall integrity, in addition to significant cellular (rounded cells) and extracellular edema (separated muscle fibers). (C) PJ34 treated skeletal muscle tissue at 24 hours reperfusion, shows fewer necrotic fibers, mild edema, and only slight loss of polygonal shape with preserved nuclear architecture (bar = 50 μ m).

(bottom). Skeletal Muscle Fiber Injury: There was a significant decrease in the percentage of injured fibers in the PJ34 treated group compared to the LR (* p = .0008).

Figure 2. Representative Immunoblot of PAR Activity at 7 hours IR

The upper panel represents a western blotting image of PARP activity evaluated at 7 hours IR by detection of poly ADP ribosylated proteins (PAR) detection in the hind limb muscle tissue extract from PJ34, LR and sham treated diabetic mice. . While uninjured mice (sham) had minimum PAR expression, the LR group had significant PAR formation that was markedly reduced in the PJ34 treated animals. The lower panel represents the Ponceau S staining of the same membrane as evidence of equal total protein loading of the western blotting gel. **Semi-quantitative analysis of PARP** activity at 1, 7 and 24 hours IR is presented in Figure 3A.

Figure 3.

A. Percent PARP Activity at 1, 7 and 24 hours IR: PARP activity increased immediately following one hour IR in the LR treated group. PARP activity continues to increase and reached significantly higher levels by 24 hours reperfusion compared to 1 hour reperfusion (**p < 0.05). PJ34 treatment did not ameliorate PARP activity following 1 hour reperfusion however, it significantly reduced PARP activity by 7 and 24 hours reperfusion (*p < 0.05) compared to mice treated with LR (Data expressed as percent PARP activity in the Sham controls).

B. PARP-1 Protein Expression: At 24 hours IR, there was no significant difference in PARP-1 protein expression in PJ34 and LR treated mice.

Figure 4. Immunohistochemical Localization of PARP Activity

Immunostaining of PAR in hind limb skeletal muscle tissue exhibited increased detection of PARP activity localized to the peripheral nuclei of the skeletal muscle fibers in LR and PJ34 treated mice subjected to IR (solid black arrows panels C and D) compared to sham (panel B). In addition, in LR treated mice, there is evidence of PAR activity in intravascular and interstitial inflammatory cells (dotted arrows panel C and D). There was no evidence of positive immunostaining for PAR in the absence of the primary antibody (panel A).

Figure 5. GAPDH during Ischemia Reperfusion

A. Representative Immunoblotting of GAPDH at 24hrs reperfusion. The upper panel shows the 37kDa bands corresponding to GAPDH and the lower panel shows the protein loading revealed with Ponceau S staining of the same membrane. **B. Effect of PJ34 on total GAPDH protein levels:** There was no significant difference in the GAPDH protein expression at 1 or 7 hours reperfusion. By 24 hours reperfusion there a slightly lower percent of GAPDH protein in the PJ34 treated group.

Figure 6. GAPDH Activity During Ischemia Reperfusion

GAPDH activity was significantly higher in the hindlimbs skeletal muscles extracts from the PJ34 treated mice at 24 hours reperfusion (* $p < 0.01$).

Figure 7. Ribosylation of GAPDH

A. Immunoblotting: Detection of poly ADP Ribosylated GAPDH with immunoprecipitation at 24 hours IR: immunoprecipitated GAPDH protein was highly ribosylated at 24 hours reperfusion in the hindlimb skeletal muscle protein lysates of the LR group. GAPDH ribosylation was markedly diminished in the PJ34 group.

B. Quantitative Analysis: Hindlimb Skeletal muscle GAPDH ribosylation significantly increased in the LR group following IR compared to sham * $p < 0.05$. PJ34 treatment markedly decreased GAPDH ribosylation compared to LR ** $p < 0.01$.

Figure 8. Immunofluorescent subcellular detection of PAR (Cy3) and GAPDH (FITC) in hindlimb skeletal muscle following 24 hours IR

White triangles indicate GAPDH nuclear detection in sham, LR and PJ34 muscle sections (A, D, and G respectively). White arrows point to PAR staining in sham, LR and PJ34 groups muscle sections in (B, E, and H respectively). Overlay merged images of GAPDH and PAR staining indicated by the orange arrows in sham, LR and PJ34 groups muscle sections in (C, F, and I respectively). The top panel shows minimal PAR expression in the sham group (A, B, C) with GAPDH staining primarily localized to the sarcoplasm. In the middle panel, IR dramatically enhanced PAR staining in both the nucleus and sarcoplasm with nuclear relocalization of GAPDH which appeared to be ribosylated (red arrow image F). PJ34 dramatically reduced ribosylated nuclear localized GAPDH (orange arrow image I). (Scale bars = 100 μ m)

Table 1

Effect of PJ34 Treatment on Inflammatory Mediators

	1 hour reperfusion		7 hours reperfusion		24 hours reperfusion	
	LR	PJ34	LR	PJ34	LR	PJ34
KC pg/mg total protein	8.57 ± 0.8	7.42 ± 0.6	79.90 ± 12.8	47.56 ± 2.6 [*]	24 ± 7	15.0 ± 3.6
MPO ng/mg total protein	9.67 ± 0.5	13.46 ± 1.5	90.50 ± 7.0	31.87 ± 7.5 ^{**}	247.4 ± 21	197.8 ± 42.6 ⁺
IL-6 pg/mg total protein	13.32 ± 1.0	11.63 ± 0.8	29.96 ± 3.0	20.97 ± 2.7	7.5 ± 1	6.8 ± 0.5

± SD

^{*} p = 0.02 vs LR^{**} p = 0.004 vs LR⁺ p < 0.001 vs 1 hour reperfusion

Table 2

Effect of PJ34 Treatment on Markers of Tissue Metabolism

	1 hour reperfusion		7 hours reperfusion		24 hours reperfusion	
	LR	PJ34	LR	PJ34	LR	PJ34
Lactate $\mu\text{g}/\text{gram tissue}$	356.3 $\pm$ 38.6	255.3 $\pm$ 22.5 [*]	257.8 $\pm$ 37.3	208.7 $\pm$ 31.8	229.5 $\pm$ 104	375.7 $\pm$ 48.3 ^{**}
ATP nmol/mg tissue	0.467 $\pm$ 0.1	0.426 $\pm$ 0.1	0.595 $\pm$ 0.2	0.437 $\pm$ 0.2	0.292 $\pm$ 0.1	0.835 $\pm$ 0.3 ⁺

$\pm$ SD

^{*} p = 0.05 vs LR

^{**} p = 0.004 vs LR

⁺ p = 0.04 vs LR