PAPER

Polyadenosine Diphosphate–Ribose Polymerase Inhibition Modulates Skeletal Muscle Injury Following Ischemia Reperfusion

Hong T. Hua, MD; Hassan Albadawi, MD; Fateh Entabi, MD; Mark Conrad, MD; Michael C. Stoner, MD; Bryan T. Meriam, AB; Ramses Sroufe, AB; Stuart Houser, MD; Glenn M. LaMuraglia, MD; Michael T. Watkins, MD

Hypothesis: Polyadenosine diphosphate–ribose polymerase (PARP) has been implicated as a mediator of inflammation and tissue necrosis in murine models of human stroke and myocardial infarction. This study was designed to determine whether PARP modulates skeletal muscle injury and cytokine-growth factor levels during ischemia-reperfusion.

Design: Prospective controlled animal study.

Setting: Medical school–affiliated university hospital.

Interventions: Mice were divided into 2 groups—treated (PJ) and untreated; all mice were subjected to unilateral hind limb tourniquet ischemia followed by 4 or 48 hours of reperfusion. In treated mice, PJ34, an ultrapotent-specific PARP inhibitor was given immediately before ischemia and prior to reperfusion. A group of PARP-1 knockout mice ($PARP^{-/-}$) were also subjected to hind limb ischemia followed by 48 hours of reperfusion.

Main Outcome Measures: After ischemia-reperfusion, muscle was harvested for measurement of edema, viability, cytokine, and vascular endothelial growth factor content.

Results: The PJ34-treated mice had increased skeletal muscle viability when compared with the untreated mice after 4 and 48 hours of reperfusion ($P<.01$). Viability between $PARP^{-/-}$ and PJ34-treated mice were similar at 48 hours of reperfusion ($P>.05$), and it exceeded that of untreated mice ($P<.01$). Tissue edema was unaltered by PARP inhibition. Tissue levels of cytokine were only different ($P<.05$) in PJ34-treated vs untreated mice at 48 hours of reperfusion. Vascular endothelial growth factor levels in PJ34-treated mice were markedly reduced when compared with untreated mice only after 4 hours of reperfusion ($P<.01$), and in $PARP^{-/-}$ mice ($P<.01$) at 48 hours of reperfusion.

Conclusions: Polyadenosine diphosphate–ribose polymerase modulates skeletal muscle viability, cytokine and vascular endothelial growth factor synthesis during reperfusion. Polyadenosine diphosphate–ribose polymerase inhibition may represent a novel method to modulate skeletal muscle ischemia-reperfusion injury.

Arch Surg. 2005;140:344-351

Author Affiliations: Division of Vascular and Endovascular Surgery (Drs Hua, Albadawi, Entabi, Conrad, Stoner, LaMuraglia, and Watkins and Messrs Meriam and Sroufe) and the Department of Pathology (Dr Houser), Massachusetts General Hospital, Harvard Medical School, and the Veterans Administration Boston Healthcare System (Dr Watkins), Boston.

ACUTE LOWER EXTREMITY ischemia represents a significant cause of morbidity and mortality in the United States with an annual incidence of 2 per 10 000 population, overall mortality of greater than 25%, and an amputation rate of 20% in survivors.[1] Limb loss usually results from irreversible tissue injury associated with either prolonged ischemia or massive reperfusion injury. Skeletal muscle represents about 76% of the mass of the lower extremities, which are often subjected to ischemia-reperfusion (I-R) following aneurysm surgery, arterial bypass surgery for atherosclerotic disease, and peripheral embolectomy. Notable changes associated with skeletal muscle I-R include edema, myonecrosis, nerve damage, and intravascular thrombosis. Experimental therapies to ameliorate skeletal muscle reperfusion injury have been directed against complement,[2] cytokines (KCs),[3] neutrophils,[4] tissue thrombosis,[5] reactive oxygen metabolites,[6] and a variety of vascular mediators.[6-8]

See Invited Critique at end of article

Recent studies of myocardial I-R indicate that inhibitors of polyadenosine diphosphate (ADP)–ribose polymerase (PARP) may modulate tissue injury during myocardial reperfusion.[9-11] Polyadenosine diphosphate–ribose polymerase is a ubiquitous DNA repair enzyme, constitu-

tively expressed in the cellular nuclei of a variety of tissues and organs. The activity of the enzyme is stimulated by DNA single-strand breaks, as seen during I-R injury. This complex enzyme contains a DNA-binding domain, an automodification domain, and a catalytic domain. Polyadenosine diphosphate–ribose polymerase transfers ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) to nuclear proteins. This transfer results in the formation of nicotinamide, which inhibits PARP activity. Under conditions of excessive PARP activation, nicotinamide is recycled into NAD^+ in an energy-consuming metabolic cycle that rapidly depletes intracellular adenosine triphosphate (ATP),[12] thereby exacerbating energy crisis during ischemia. Free radicals and reactive oxygen metabolites generated during reperfusion cause additional DNA strand breaks and further activation of PARP.[13] Polyadenosine diphosphate–ribose polymerase activity has also been shown to be involved in the regulation of inflammatory processes, being functionally associated with important transcription factors, notably transcription factor nuclear factor κ B.[12]

The current studies were designed to determine whether the water-soluble PARP inhibitor PJ34 could modulate skeletal muscle I-R injury in a murine model. Markers of skeletal muscle responses to injury include the neutrophil CXC chemokine KC (also known as "growth oncogene-1") and the vascular endothelial growth factor (VEGF). To date, KC has been implicated in tissue injury following myocardial, hepatic, and renal I-R.[14-16] The VEGF, also known as the "vascular permeability factor," plays a key role in mediating physiologic and pathologic angiogenesis in response to ischemic stress.[17-19] The VEGF induces vasodilation and stimulates proliferation, migration, and survival of endothelial cells.[20] The VEGF has been shown to induce mobilization of endothelial cell precursors that target ischemic tissue and differentiate into vascular endothelial cells.[21,22] More recent evidence suggests that VEGF may enhance myoblast survival during ischemic stresses, primarily through antiapoptotic pathways.[23]

METHODS

CONTROLLED TENSION TOURNIQUET

The controlled-tension tourniquet was used to induce reproducible levels of hind limb I-R in the proximal hind limb of the mouse, as previously described.[24] The controlled-tension tourniquet was mounted on a table, which was preheated to maintain the mouse's monitored temperature at 37°C. Ischemia was confirmed using laser Doppler imaging (Moor Instruments Inc, Wilmington, Del) as previously described. Reperfusion is initiated by releasing tension on the controlled-tension tourniquet.

ANIMAL PROTOCOL

Animal care and experimental procedures complied with *Principles of Laboratory Animal Care*.[25] 129S1/SvImJ (wild-type) mice (Jackson Laboratory, Bar Harbor, Me) were anesthetized using intraperitoneal administration of 60 to 90 mg/kg of pentobarbital. Treated animals (PJ, n=20) received the PARP inhibitor *N*- (6-oxo-5, 6-dihydropenanthridin-2-yl)-2-(*N*, *N*-dimethylamino) acetamide (PJ34; Calbiochem, Spring Valley Calif, 15 mg/kg in 0.1 mL of isotonic sodium chloride intraperitoneally) immediately prior to ischemia and again prior to reperfusion. Untreated mice (UN, n=20) and 129S-Adprt1^{tm1Zqw} ($PARP^{-/-}$) mice (n=10) were given 0.1 mL of isotonic sodium chloride intraperitoneally immediately prior to ischemia and again prior to reperfusion. Thirty minutes after the induction of anesthesia, the controlled-tension tourniquet was used to induce unilateral hind limb ischemia for 3 hours of ischemia followed by 4 or 48 hours of reperfusion. Mice (n=10 PJ and 10 UN) remained anesthetized throughout the duration of ischemia and the initial early reperfusion (4-hour) interval. Mice studied for 48-hour reperfusion (n=10 PJ, 10 UN, and 10 $PARP^{-/-}$) were allowed to recover from anesthesia after the initial 4 hours of reperfusion and were returned to their cages. After 4 or 48 hours of reperfusion, the animals were killed (200 mg/kg of pentobarbital, intraperitoneally). Ischemia-reperfusion and contralateral limbs were then harvested and the skin removed from each limb for KC and tissue viability assays (see "Estimation of Tissue Viability" section). Sham animals were treated exactly as the experimental animals except that no tension was applied to the tourniquet.

ESTIMATION OF TISSUE VIABILITY

Tissue viability was estimated by the reduction of a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium [MTT]) to water-insoluble colored formazan crystals by electron carriers and oxidative enzymes in the mitochondria of viable tissue. At the end of the experiment the limbs were harvested, the skin removed, and the tissue cut into 3 pieces to increase surface area and tissue uptake of the tetrazolium salt. Each piece was weighed and placed in a small tube with 3 mL of phosphate-buffered solution (PBS) (pH 7.4) supplemented with 300 µL of 1-mg/mL MTT (Sigma, St Louis, Mo). The samples were then incubated for 3 hours at 37°C in the dark on a rotating mixer. The samples were then removed, washed with distilled water, and blotted dry. The water-insoluble formazan salt was extracted in 3 mL of 2-propanol overnight at 37°C in the dark on a rotating mixer. Aliquots of 200 µL were removed and the optical density (OD) was determined at 570 nm using a microplate reader. The tissue samples were then dried at 90°C for 24 hours. The viability index was then expressed as OD570 relative to dry tissue weight in the experimental vs the nonischemic contralateral limb.

TISSUE KC AND VEGF LEVELS

Hind limb skeletal muscle samples were snap frozen in liquid nitrogen immediately after harvest and stored at –80°C until analysis. Muscle samples were homogenized with a polytron homogenizer in a test tube containing radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Sigma P-8340) and placed on ice for 30 minutes. The samples were then sonicated for 20 seconds and incubated on ice for 30 minutes. The homogenized sample was transferred to a microcentrifuge tube and centrifuged at 10 000*g* for 10 minutes. The supernatants were frozen in aliquots at -80°C until analysis. The levels of KC and VEGF were measured with enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minn). The enzyme-linked immunosorbent assay plates were read with a plate reader (Spectromax-250; Molecular Devices, Sunnyvale, Calif). Values were extrapolated off the standard curve and normalized to the total protein concentration, which was determined with the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, Ill).

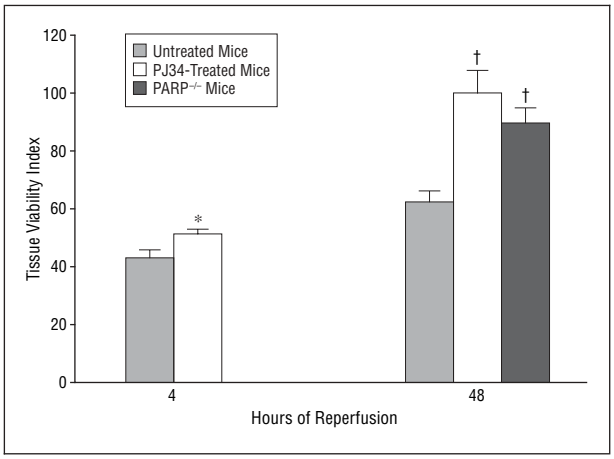

Figure 1. Skeletal muscle viability. PJ34 treatment (See "Animal Protocol" subsection of "Methods" section for expansion of PJ34.) resulted in modest improvement in tissue viability after 4 hours of reperfusion (asterisk indicates $P=.01$ vs untreated). Tissue viability in both PJ34-treated and polyadenosine diphosphate–ribose polymerase ($PARP^{-/-}$) knockout mice was significantly greater than untreated mice after 48 hours of reperfusion (dagger indicates $P<.05$ vs untreated).

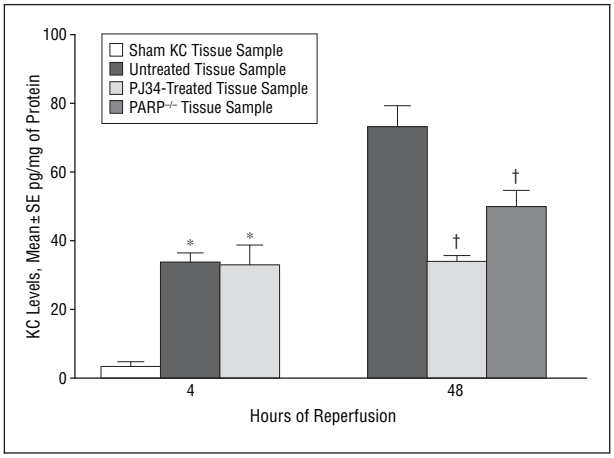

Figure 2. Tissue cytokine (KC) levels following reperfusion. The KC levels were increased 10- and 20-fold (asterisk indicates $P<.001$ vs sham) after 4 and 48 hours of reperfusion, respectively. Tissue KC levels in polyadenosine diphosphate–ribose polymerase ($PARP^{-/-}$) knockout and PJ34-treated (See "Animal Protocol" subsection of "Methods" section for expansion of PJ34.) mice contained decreased KC levels after 48 hours but not after 4 hours of reperfusion (dagger indicates $P<.01$ vs untreated at 48 hours).

DETERMINATION OF TISSUE EDEMA

Skeletal muscle edema was quantified by measuring tissue wet-dry weight ratio. Immediately after harvest, tissue samples were blotted, weighed, and placed in a drying oven at 55°C until a constant weight was obtained. Muscle edema is quantified by measuring the wet-dry weight ratio.

IMMUNOHISTOCHEMISTRY

Harvested mouse hind limbs were fixed in 10% buffered formalin, decalcified, and embedded in paraffin. Four- to 6-µm-thick transverse sections were mounted on positively charged slides and subsequently deparaffinized in xylene and rehydrated in graded alcohols. The slides were incubated for 5 minutes in 1% hydrogen peroxide, washed in PBS, then blocked for 1 hour with 1% serum at room temperature. These slides were washed again, then blocked with biotin reagent for 15 minutes. Slides were rinsed with PBS and incubated with either VEGF goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, Calif) at 1:200 dilution or antimouse KC goat polyclonal IgG (R&D Systems) at 1 µg/mL in PBS overnight at room temperature, washed 3 times in PBS, and incubated for 30 minutes with biotin-conjugated secondary antibody (1 µg/mL in 1% blocking serum). The slides were rinsed 3 times with PBS and developed with 3,3′-diaminobenzindine chromogen reagent solution (R&D Systems) according to the manufacturer's instructions. Slides were counterstained with hematoxylin and examined by light microscopy.[26]

STATISTICAL ANALYSIS

Statistical analysis was performed with Instat (GraphPad, San Diego, Calif). Skeletal muscle tissue edema and VEGF-KC levels in treated and untreated mice after 4 and 48 hours of reperfusion were compared with each other and to sham using analysis of variance (ANOVA) with Tukey-Kramer posttests. Skeletal muscle viability in treated and untreated mice was compared using unpaired *t* test. Skeletal muscle wet-dry weight ratio, KC and VEGF levels in wild type sham, and $PARP^{-/-}$ sham mice were also compared using unpaired *t* test.

RESULTS

SKELETAL MUSCLE VIABILITY

PJ34 significantly increased skeletal muscle viability after 4 hours of reperfusion (mean±SE, 51.5%±1.5% for PJ-treated vs 43.5%±2.1% for UN mice, $P<.01$). By 48 hours of reperfusion, tissue viability in untreated mice was significantly less than both the PARP-$1^{-/-}$ and PJ34-treated mice, which recovered to near 100% viability (mean±SE, 62.6%±3.7% for UN mice vs 89%±5% for $PARP^{-/-}$ and 100%±8% for PJ-treated mice, $P<.05$, **Figure 1**). These results are consistent with the hypothesis that PARP modulates tissue injury following hind limb I-R. The specific action of the PARP inhibitor PJ34 is supported by the fact that the viability of the PJ34-treated mice and $PARP^{-/-}$ mice is indentical at 48 hours of reperfusion.

KC LEVELS FOLLOWING REPERFUSION

After 4 hours of reperfusion in PJ34-treated and UN mice, skeletal muscle tissue levels of KC were markedly increased when compared with sham mice (mean±SE, 32.8±5.8 pg/mg of protein for PJ-treated , 33.5±2.8 for UN, vs 3.5±1.1 for sham mice, $P<.001$, **Figure 2**) but not significantly different from one another (32.8±5.8 for PJ-treated vs 33.5±2.8 for UN mice, $P>.05$, Figure 2). After 48 hours of reperfusion in PJ34-treated and $PARP^{-/-}$ mice KC levels were markedly decreased when compared with UN mice (33.9±1.6 pg/mg of protein for PJ-treated, 50.1±4.7 for $PARP^{-/-}$ vs 73±6 for UN mice, $P<.01$). The KC levels in $PARP^{-/-}$ sham and wild-type sham mice were not different (3.5±1.1 wt sham vs 6.5±1.6 pg/mg of protein, $P=.17$). These findings suggest that the effect of PJ34 on skeletal muscle chemokine accumulation during reperfusion is not evident until after 4 hours.

VEGF LEVELS FOLLOWING REPERFUSION

After 4 hours of reperfusion, tissue VEGF levels in PJ34-treated and UN mice were both greater than in sham mice (mean±SE, 31±3.2 pg/mg of protein for PJ-treated, 46.1±3.5 for UN mice, vs 16±1.4 for sham mice, $P<.001$, **Figure 3**) and significantly different from one another (31±3.2 pg/mg of protein for PJ-treated vs 46.1±3.5 for UN mice, $P<.01$, Figure 3). After 48 hours of reperfusion in PJ34-treated and UN mice, skeletal muscle levels of VEGF were not significantly different from one another but were significantly greater than levels found in PARP$^{-/-}$ mice (mean±SE, 42.3±3.1 pg/mg of protein for UN, 40.8±4.3 for PJ mice vs 25.2±4.2 for PARP$^{-/-}$ mice, $P<.05$). The VEGF levels in wild-type sham mice and PARP$^{-/-}$ mice were not different (mean±SE, 16±1.4 for sham vs 14.5±1.7 pg/mg of protein for PARP$^{-/-}$ mice, $P=.60$). These results indicate that short-term administration of PJ34 decreased VEGF levels only during 4 hours of reperfusion, whereas genetic deletion of the PARP-1 enzyme did effectively inhibit increased VEGF levels even after 48 hours of reperfusion.

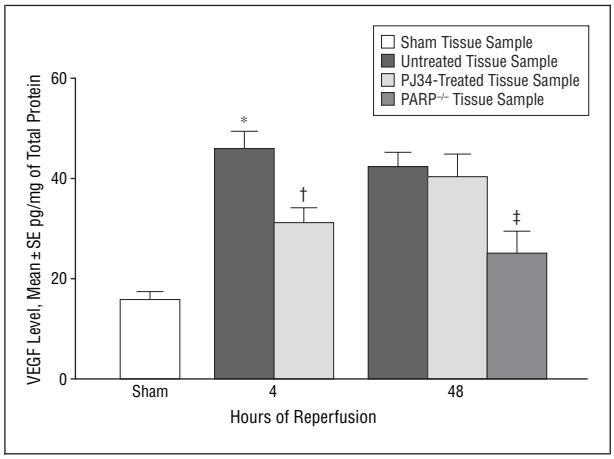

Figure 3. Tissue levels following reperfusion. Vascular endothelial growth factor (VEGF) levels were significantly increased in reperfused muscle of untreated and PJ34-treated (See "Animal Protocol" subsection of "Methods" section for expansion of PJ34.) mice (asterisk indicates $P<.01$ vs sham) at 4 hours of reperfusion. PJ34 treatment significantly reduced tissue VEGF levels compared with untreated mice at 4 hours (dagger indicates $P<.01$ vs untreated) but not at 48 hours of reperfusion. At 48 hours of reperfusion, only polyadenosine diphosphate–ribose polymerase (PARP$^{-/-}$) knockout mice had VEGF levels that were significantly reduced (double dagger indicates $P<.05$ vs treated and untreated mice).

TISSUE EDEMA

After 4 and 48 hours of reperfusion, UN mice developed significant levels of edema that were greater than those for sham mice (6.0±0.2 for UN mice after 4 hours, 5.8±0.1 for UN after 48 hours vs 4.5±0.2 for sham, $P<.001$, **Figure 4**). PJ34 treatment did not change the degree of tissue edema detected in reperfused muscle after 4 and 48 hours of reperfusion ($P>.05$). After 48 hours of reperfusion, PARP$^{-/-}$ mice also developed significant levels of edema, which were greater than those for sham mice (6.4±0.2-PARP$^{-/-}$ vs 4.5±0.2 for sham, $P<.001$). The wet-dry weight ratio in sham vs PARP$^{-/-}$ sham mice was not different (4.5±0.2 wild-type for sham vs 4.8±0.4 PARP$^{-/-}$ for sham, $P=.45$). These results suggest that the PARP enzyme does not contribute to tissue edema following I-R.

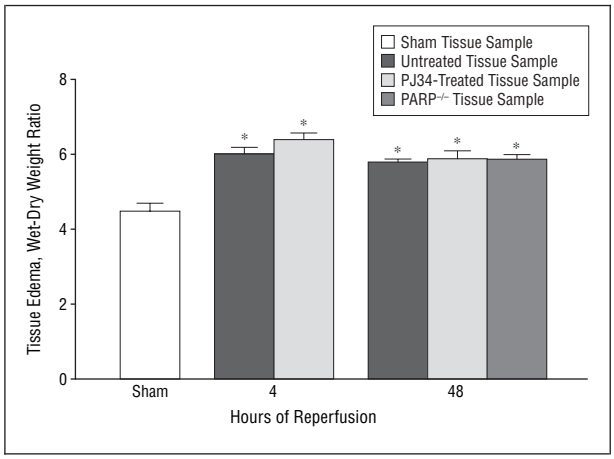

Figure 4. Tissue edema following reperfusion. Skeletal muscle edema was significantly increased (asterisk indicates $P<.01$ vs sham) during reperfusion, but not altered by pharmacologic treatment with PJ34 (See "Animal Protocol" subsection of "Methods" section for expansion of PJ34.) or genetic deletion of PARP-1 knockout (PARP$^{-/-}$).

IMMUNOHISTOCHEMISTRY

The pattern of tissue staining for KC was markedly different in untreated and PJ34-treated mice. In untreated mice (**Figure 5**A), there was variably intense staining of muscle bundles whereas in the treated animals, the staining was much less intense (Figure 5B). The VEGF staining of muscle fibers in untreated mice (**Figure 6**A) was similar to that for treated mice (Figure 6B); staining was clearly less in nonischemic tissue (Figure 6C). These qualitative findings directly correlate with the quantitative assessment of tissue KC and VEGF levels via enzyme-linked immunosorbent assay.

COMMENT

The major finding in this article indicates PARP modulates skeletal muscle tissue injury, KC, and VEGF synthesis following I-R. The water-soluble PARP inhibitor PJ34 salvaged skeletal muscle viability following reperfusion. Tissue viability in this article and other studies[27,28] of isolated cells and whole skeletal muscle tissues[29] was measured using a standard reproducible biochemical assay of mitochondrial activity. Mitochondrial activity was estimated by the reduction of a tetrazolium salt to water-insoluble colored formazan crystals by electron carriers and oxidative enzymes in the mitochondria of viable tissue.[30-32] Testing mitochondrial function in the setting of skeletal muscle I-R is appropriate since in vitro studies with cardiac myoblasts have demonstrated that peroxynitrite and hydrogen peroxide (often present in cells during reperfusion) cause PARP activation that leads to decreased mitochondrial respiration.[33] As it is unlikely that PJ34 mediated skeletal muscle regeneration from 55% to 100% after only 48 hours[34] (Figure 1), the improved tissue viability observed at 48 hours represents reversible mitochondrial

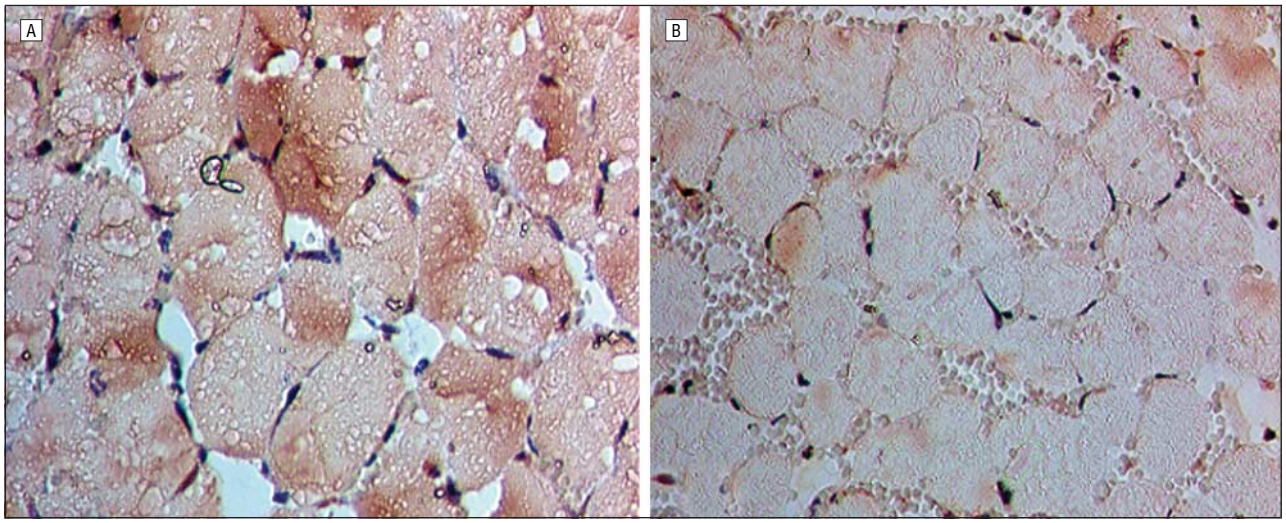

Figure 5. Tissue KC immunohistochemistry after 48 hours of reperfusion (original magnification ×40). A, A diffuse granular appearance of KC staining is present in muscle bundles. B, Cytokine staining is much less intense in PJ34-treated mice. (See "Animal Protocol" subsection of "Methods" section for expansion of PJ34.)

dysfunction. Data in this report are consistent with in vivo and in vitro studies in which investigators have demonstrated that pharmacologic inhibition of PARP prevented decreases in mitochondrial respiration caused by oxidative stresses, hypoxia, and reoxygenation. Furthermore, PJ34[13] and other PARP inhibitors[35] prevent depletion of myocardial high-energy phosphates in cardiac tissue subjected to I-R. The specificity of the effect of PJ34 on tissue viability is bolstered by a similar level of mitochondrial activity in the PARP-$1^{-/-}$ mice noted at 48 hours of reperfusion (Figure 1).

Levels of KC in tissue KC were analyzed because of its potent neutrophil chemoattractant activity, the crucial role neutrophils play in the development of I-R injury,[36,37] and its functional relationship to human interleukin 8.[38] After 4 hours of reperfusion, KC levels in skeletal muscle were 10 times the level detected in sham animals (Figure 2). PJ34 did not mediate change in tissue levels of KC at 4 hours of reperfusion even though tissue viability was significantly improved at the same time (Figure 1). This finding suggests that the improvement in viability observed at 4 hours of reperfusion was not related to an effect that PJ34 might have on KC-mediated inflammation. The assessment of KC levels in skeletal muscle was extended to a 48-hour interval because of intriguing and compelling findings of others[39,40] that demonstrated that neutrophil activation and the inflammatory process accompanying skeletal muscle reperfusion clearly persist to at least 24 hours. Indeed, by 48 hours of reperfusion, KC tissue levels in UN mice were 20 times the levels detected in sham animals and were accompanied by an ongoing decrease in tissue viability (62%). In contrast to the effects observed at 4 hours of reperfusion, by 48 hours PJ34 treatment was associated with a significant decrease in KC tissue levels (Figure 2). These data are consistent with the hypothesis that PJ34 decreases the proinflammatory response to skeletal muscle I-R injury. Since there was no difference in the KC levels in the PJ34-treated and PARP-$1^{-/-}$ mice, these experiments suggest that the short-term administration of PJ34 and its brief serum half-life (30 minutes) did not detract from its ability to modulate tissue viability even after 48 hours of reperfusion.

In contrast to the logarithmic changes in KC tissue levels following skeletal muscle I-R (Figure 2), the relative increase in VEGF levels in tissue in response to reperfusion was quite modest (ie, 2- to 3-fold, Figure 3). While 4 and 48 hours of skeletal muscle reperfusion were all characterized by significantly increased levels (2- to 3-fold) of VEGF compared with sham animals, treatment with PJ34 transiently decreased the VEGF response only at 4 hours. In a study of porcine latissumus dorsi flaps subjected to I-R, VEGF levels were found to be elevated preferentially in muscle vs skin.[41] Administration of exogenous VEGF has been shown to improve survival of gracilis muscle free flaps in the rat.[42] The VEGF antagonists were found to decrease both brain edema and loss of cortical tissue in a rat stroke model[43] whereas in a brain injury model, VEGF antagonism actually led to increased tissue injury.[44]

Despite these interesting observations, the physiologic significance of increased tissue levels of VEGF in skeletal muscle following acute reperfusion is unclear. Based on our findings of decreased levels of VEGF in tissue associated with PJ34 treatment and improved tissue viability only at 4 hours of reperfusion, it is possible that VEGF may contribute to tissue injury only during early reperfusion. However, by 48 hours of reperfusion, there is no evidence to suggest that VEGF levels correlate with either ongoing skeletal muscle injury or recovery. Since VEGF levels in PARP$^{-/-}$ mice were markedly decreased when compared with those of PJ34-treated mice at 48 hours, it is unlikely that the short-term administration of PJ34 had any long-term effect on VEGF levels in tissue. This conclusion is supported by the fact that in the PARP-$1^{-/-}$ animals, PARP-1 activity is effectively absent throughout the entire experimental protocol, uninfluenced by the serum half-life of a drug such as PJ34.

Ischemia-reperfusion resulted in a marked increase in tissue edema after 4 and 48 hours of reperfusion

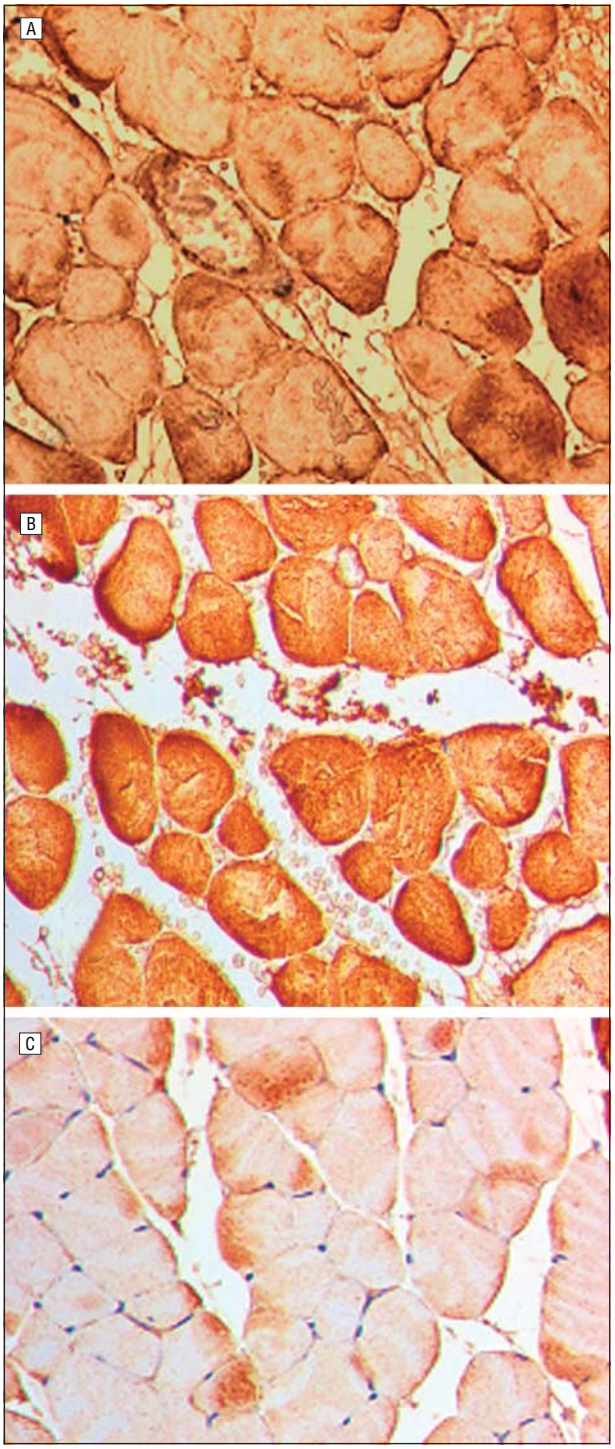

Figure 6. Vascular endothelial growth factor (VEGF) immunohistochemistry after 48 hours of reperfusion (original magnification×40). Diffuse staining for VEGF is clearly visible in the untreated (A) and PJ34-treated mice (B). (See "Animal Protocol" subsection of "Methods" section for expansion of PJ34.) C, While the pattern of VEGF staining in the untreated and PJ34-treated mice was similar, it was clearly increased over that of tissue not subjected to ischemia reperfusion.

(Figure. 4). Despite a marked increase in tissue viability associated with PJ34 treatment at 48 hours, this improvement was not accompanied by a decrease in edema. This finding is in contrast to experimental protocols in which potential therapeutic agents such as vitamin C, inosine, or glycine did successfully improve both tissue viability and decrease edema in rabbit, murine, and canine models.[45-47] Our findings do suggest that decreased edema is not a prerequisite for improved tissue viability in mice following I-R. The angiogenic growth factor VEGF was originally known as "vascular permeability factor." Since tissue edema has been linked with VEGF administration in models of experimental angiogenesis,[48,49] a potential role for VEGF in postischemic tissue edema was considered. However, at no experimental interval was the level of VEGF in tissue coincident with a decrease in the degree of tissue edema.

Clinical Relevance

These experiments were designed to determine whether an inhibitor of PARP modulates the outcome of skeletal muscle I-R injury. The therapeutic protocol in this report is similar to a widely used preischemic or prereperfusion treatment scenario.[3,29,56-58] Despite the experimental usefulness of this approach, an important step toward its application to the clinical arena will require advances in methods for drug delivery that facilitate their administration to limbs during ischemia, prior to reperfusion. Until such technology exists, experimental treatment protocols remain useful in delineating the biochemical pathways that influence the outcome of I-R injury. Since PARP is a ubiquitous enzyme in mammalian cells whose activity simultaneously influences a number of different physiologic pathways on a metabolic and transcriptional level, PARP inhibition may be a promising therapeutic tool for humans suffering from a variety of systemic and focal ischemic complications. The physiologic consequences of PARP inhibition may exceed that of antibodies directed toward one element of the stress response or a single anti-KC regimen in the treatment of local and systemic reperfusion injury. This is important because clinical trials in humans suffering from complex problems such as multisystem organ failure or sepsis suggests that a focused "magic bullet" directed against one component of the stress response will be useful in animal models but clinically ineffective.[59]

CONCLUSIONS

This article provides a unique perspective on how the water-soluble PARP inhibitor PJ34 modulates skeletal muscle viability and the pattern of tissue KC/growth factors following acute I-R. PJ34, unlike other PARP inhibitors does not have any known biological activity. It is likely that the primary target for PJ34 and other PARP inhibitors is skeletal muscle and endothelial cells, which are rich in PARP.[50,51] Granulocytes, the primary target cells for KC, do not contain the PARP enzyme.[52] These findings agree with the effects of PARP inhibition on myocardium[13,53] and brain tissue.[12] Since PARP is an abundant evolutionally conserved protein, which plays a role in physiologic housekeeping at the level of gene repair, transcription, and cell cycling, some concern exists regarding the consequences of PARP inhibition in humans. Chronic PARP inhibition and PARP deficiency have been associated with increased sister chromatid exchange,[54] which

may indicate a risk of malignant transformation. However, PARP deficiency has not been associated with increased frequency of malignancies.[55] Ongoing work in this field will likely yield promising information regarding the therapeutic utility of PARP inhibitors.

Accepted for Publication: December 14, 2004.
Correspondence: Michael T. Watkins, MD, Division of Vascular and Endovascular Surgery, Massachusetts General Hospital, 15 Parkman St, Suite 458, Boston, MA 02114 (mtwatkins@partners.org).
Funding/Support: This study was supported in part by a Merit Award from the Research Service of the Veterans Administration, the Veterans Affairs Department of Defense Combat Casualty Care Project, and the Department of Surgery, Division of Vascular and Endovascular Surgery, Massachusetts General Hospital.
Previous Presentation: This study was presented at the 85th Annual Meeting of the New England Surgical Society; October 1, 2004; Montreal, Quebec; and is published after peer review and revision. The discussions that follow this article are based on the originally submitted manuscript and not the revised manuscript.
Acknowledgment: Dr Hua received the award for the best resident presentation during the New England Surgical Society meeting.

REFERENCES

1. Jivegard L, Holm J, Shersten T. Acute limb ischemia due to arterial embolism or thrombosis: influence of limb ishemia versus pre-existing cardiac disease on postoperative mortality rate. *J Cardiovasc Surg (Torino)*. 1988;29:32-36.
2. Kyriakides C, Austen WG Jr, Wang Y, Favuzza J, Moore FD Jr, Hechtman HB. Neutrophil mediated remote organ injury after lower torso ischemia and reperfusion is selectin and complement dependent. *J Trauma Injury Infect Crit Care*. 2000;48:32-38.
3. Seekamp A, Warren JS, Remick DG, Till GO, Ward PA. Requirements for tumor necrosis factor-alpha and interleukin-1 in limb ischemia/reperfusion injury and associated lung injury. *Am J Pathol*. 1993;143:453-463.
4. Hernandez L, Grisham M, Twohig B, Arfors K, Harlan J, Granger D. Role of neutrophils in ischemia-reperfusion induced microvascular injury. *Am J Physiol*. 1987; 253:H699-H703.
5. Hobson RW II, Neville R, Watanabe B, Canady J, Wright JG, Belkin M. Role of heparin in reducing skeletal muscle infarction in ischemia-reperfusion. *Microcirc Endothelium Lymphatics*. 1989;5:259-276.
6. Koromopilias A, Chen L, Seaber A, Urbaniak J. Studies of ischemia-reperfusion injury in skeletal muscle: efficacy of 21-aminosteroids on microcirculation and muscle contraction after an extended period of warm ischemia. *J Orthop Res*. 1997;15:512-518.
7. Barker JE, Knight KR, Romeo R, Hurley JV, Morrison WA, Stewart AG. Targeted disruption of the nitric oxide synthase 2 gene protects against ischaemia/reperfusion injury to skeletal muscle. *J Pathol*. 2001;194:109-115.
8. Ozaki M, Kawashima S, Hirase T, et al. Overexpression of endothelial nitric oxide synthase in endothelial cells is protective against ischemia-reperfusion injury in mouse skeletal muscle. *Am J Pathol*. 2002;160:1335-1344.
9. Bowes J, McDonald MC, Piper J, Thiemermann C. Inhibitors of poly (ADP-ribose) synthetase protect rat cardiomyocytes against oxidant stress. *Cardiovasc Res*. 1999;41:126-134.
10. Bowes J, Ruetten H, Martorana PA, Stockhausen H, Thiemermann C. Reduction of myocardial reperfusion injury by an inhibitor of poly (ADP-ribose) synthetase in the pig. *Eur J Pharmacol*. 1998;359:143-150.
11. Choi DW. At the scene of ischemic brain injury: is PARP a perp? *Nat Med*. 1997; 3:1073-1074.
12. Virag L, Szabo C. The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. *Pharmacol Rev*. 2002;54:375-429.
13. Szabo G, Bahrle S, Stumpf N, et al. Poly(ADP-ribose) polymerase inhibition reduces reperfusion injury after heart transplantation. *Circ Res*. 2002;90:100-106.
14. Daemen MA, de Vries B. van't Veer C, Wolfs TG, Buurman WA. Apoptosis and chemokine induction after renal ischemia-reperfusion. *Transplantation*. 2001; 71:1007-1011.
15. Bao W, Hu E, Tao L, et al. Inhibition of Rho-kinase protects the heart against ischemia/reperfusion injury. *Cardiovasc Res*. 2004;61:548-558.
16. Lentsch AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. *Hepatology*. 1998;27:1172-1177.
17. Nakajima K, Tabata S, Yamashita T, et al. Plasma vascular endothelial growth factor level is elevated in patients with multivessel coronary artery disease. *Clin Cardiol*. 2004;27:281-286.
18. Manoonkitiwongsa PS, Schultz RL, McCreery DB, Whitter EF, Lyden PD. Neuroprotection of ischemic brain by vascular endothelial growth factor is critically dependent on proper dosage and may be compromised by angiogenesis. *J Cereb Blood Flow Metab*. 2004;24:693-702.
19. Depre C, Kim SJ, John AS, et al. Program of cell survival underlying human and experimental hibernating myocardium. *Circ Res*. In press.
20. Matsumoto T, Claesson-Welch L. VEGF receptor signal transduction. *Sci STKE*. 2001;112:RE21.
21. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow–derived endothelial cell progenitor cells. *EMBO J*. 1999;18:3964-3972.
22. Kalka C, Masuda H, Takahashi H, et al. *VEGF165* gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res*. 2000;86:1198-1202.
23. Germani A, Di Carlo A, Mangoni A, et al. Vascular endothelial growth factor modulates skeletal myoblast function. *Am J Pathol*. 2003;163:1417-1428.
24. Bonheur JA, Albadawi H, Patton GM, Watkins MT. A noninvasive murine model of hind limb ischemia-reperfusion injury. *J Surg Res*. 2004;116:55-63.
25. *Principles of Laboratory Animal Care: Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Institutes of Health; 1985. NIH publication 86-23.
26. Ishibashi N, Weisbrot-Lefkowitz M, Reuhl K, Inouye M, Mirochnitchenko O. Modulation of chemokine expression during ischemia/reperfusion in transgenic mice overproducing human glutathione peroxidases. *J Immunol*. 1999;163: 5666-5677.
27. Arnaud F. Endothelial and smooth muscle changes in the thoracic and abdominal aorta wit various types of cryopreservation. *J Surg Res*. 2000;89:147-154.
28. Newman JM, DiMaria CA, Rattigan S, et al. Relationship of MTT reduction to stimulants of muscle metabolism. *Chem Biol Interact*. 2000;128:127-140.
29. Belkin M, Wright JG, Hobson RW. Iloprost infusion decreases skeletal muscle ischemia-reperfusion injury. *J Vasc Surg*. 1990;11:77-82.
30. Dubowitz V. Muscle biopsy–technical and diagnostic aspects. *Ann Clin Res*. 1974; 6:69-79.
31. Altman FP. Tetrazolium salts and formazans. *Prog Histochem Cytochem*. 1976; 9:1-56.
32. Seidler E. The tetrazolium-formazan system: design and histochemistry. *Prog Histochem Cytochem*. 1991;24:1-86.
33. Gilad E, Zingarelli B, Salzman AL, Szabo C. Protection by inhibition of poly(ADP-ribose) synthetase against oxidant injury in cardiac myocytes in vitro. *J Mol Cell Cardiol*. 1997;29:2585-2597.
34. Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. *Physiol Rev*. 2004;84:209-238.
35. Docherty JC, Kuzio B, Silvester JA, Bowes J, Thiemermann C. An inhibitor of poly (ADP-ribose) synthetase activity reduces contractile dysfunction and preserves high-energy phosphate levels during reperfusion of the ischaemic rat heart. *Br J Pharmacol*. 1999;127:1518-1524.
36. Belkin M, LaMorte WL, Wright JG, Hobson RW II. The role of leukocytes in the pathophysiology of skeletal muscle ischemic injury. *J Vasc Surg*. 1989;10: 14-19.
37. Carden DL, Smith JK, Korthius RJ. Neutrophil mediated microvascular dysfunction in postischemic canine skeletal muscle. role of granulocyte adherence. *Circ Res*. 1990;66:1436-1444.
38. Onodera S, Nishihira J, Koyama Y, et al. Macrophage migration inhibitory factor up-regulates the expression of interleukin-8 messenger RNA in synovial fibroblasts of rheumatoid arthritis patients: common transcriptional regulatory mechanism between interleukin-8 and interleukin-1beta. *Arthritis Rheum*. 2004;50: 1437-1447.
39. Freischlag J, Hanna D. Neutrophil (PMN) phagocytosis and chemotaxis after reperfusion injury. *J Surg Res*. 1992;52:152-156.
40. Summers S, Wyatt L, Freischlag J. Persistent neutrophil activation 24 hours after ischemia and reperfusion. *J Surg Res*. 1994;56:130-133.
41. Erdmann D, Sweis R, Wong MS, et al. Vascular endothelial growth factor expression in pig latissimus dorsi myocutaneous flaps after ischemia reperfusion injury. *Plast Reconstr Surg*. 2003;111:775-780.
42. Kryger Z, Dogan T, Zhang F, et al. Effects of VEGF administration following is-

chemia on survival of the gracilis muscle flap in the rat. *Ann Plast Surg*. 1999; 43:172-178.
43. van Bruggen N, Thibodeaux H, Palmer JT, et al. VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J Clin Invest*. 1999;104:1613-1620.
44. Krum JM, Khaibullina A. Inhibition of endogenous VEGF impedes revascularization and astroglial proliferation: roles for VEGF in brain repair. *Exp Neurol*. 2003; 181:241-257.
45. Wakai A, Winter DC, Street JT, O'Sullivan RG, Wang JH, Redmond HP. Inosine attenuates tourniquet-induced skeletal muscle reperfusion injury. *J Surg Res*. 2001;99:311-315.
46. Kearns SR, Moneley D, Murray P, Kelly C, Daly AF. Oral vitamin C attenuates acute ischaemia-reperfusion injury in skeletal muscle. *J Bone Joint Surg Br*. 2001; 83:1202-1206.
47. Ascher E, Hanson JN, Cheng W, Hingorani A, Scheinman M. Glycine preserves function and decreases necrosis in skeletal muscle undergoing ischemia and reperfusion injury. *Surgery*. 2001;129:231-235.
48. Byzova TV, Goldman CK, Jankau J, et al. Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns in vivo. *Blood*. 2002;99:4434-4442.
49. Yamauchi A, Ito Y, Morikawa M, et al. Pre-administration of angiopoietin-1 followed by VEGF induces functional and mature vascular formation in a rabbit ischemic model. *J Gene Med*. 2003;5:994-1004.
50. Junod A, Jornot J, Petersen H. Differential effects of hyperoxia and hydrogen peroxide on DNA damage, polyadenosine diphosphate-ribose polymerase activity and nicotinamide adenine dinucleotide and adenosine triphosphate contents in cultured endothelial cells and fibroblasts. *J Cell Physiol*. 1989;140:177-185.
51. Grupp I, Jackson T, Hake P, Grupp G, Szabo C. Protection against hypoxia-reoxygenation in the absence of poly (ADP) ribose synthetase in isolated working hearts. *J Mol Cell Cardiol*. 1999;31:297-303.
52. Szabados E, Fischer G, Toth K, et al. Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of AZT-induced cardiomyopathy in rat. *Free Radic Biol Med*. 1999;26:309-317.
53. Liaudet L, Szabo E, Timashpolsky L, Virag L, Cziraki A, Szabo C. Suppression of poly (ADP-ribose) polymerase activation by 3-aminobenzamide in a rat model of myocardial infarction: long-term morphological and functional consequences. *Br J Pharmacol*. 2001;133:1424-1430.
54. Oikawa A, Tohda H, Kanai M, Miwa M, Sugimura T. Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges. *Biochem Biophys Res Commun*. 1980;97:1311-1316.
55. Szabo G, Liaudet L, Hagl S, Szabo C. Poly(ADP-ribose) polymerase activation in the reperfused myocardium. *Cardiovasc Res*. 2004;61:471-480.
56. Wright JG, Kerr JC, Valeri CR, Hobson RW II. Heparin decreases ischemia-reperfusion injury in isolated canine gracilis model. *Arch Surg*. 1988;123:470-472.
57. Gaines G, Welborn M, Moldawer L, Huber T, Harward T, Seeger J. Attenuation of skeletal muscle ischemia/reperfusion injury by inhibition of tumor necrosis factor. *J Vasc Surg*. 1999;29:370-376.
58. Tassiopoulos A, Carlin R, Gao Y, et al. Role of nitric oxide and tumor necrosis factor on lung injury caused by ischemia/reperfusion of the lower extremities. *J Vasc Surg*. 1997;26:647-656.
59. Baue A. Multiple organ failure, multiple organ dysfunction syndrome, and systemic inflammatory response syndrome. Why no magic bullets? *Arch Surg*. 1997; 132:703-707.

DISCUSSION

Benedict Cosimi, Boston, Mass: I have 2 questions. One is related to your model in which you gave the therapy before the ischemic insult. Typically in most surgical situations, we see the problem after the insult. Have you any data regarding treatment after the ischemic insult? The second question relates to the fact that you have 100% viability at 48 hours despite persistence of significant edema. This suggests that there is some other protective mechanism that is probably unrelated to the endothelial swelling that we have always presumed was causing the trouble. Do you have any suggestions regarding what mechanism might be involved in providing 100% viability but not resolving the edema?

Dr Hua: To answer your first question, we have not done an experiment to date in which we give PJ34 only after the ischemic insult. Certainly, this is in our future plans. For these initial experiments, we wanted to evaluate the maximal effect of PJ34, in the best-case scenario, so we gave it prior to ischemia and again just before reperfusion. In addition, the half-life of PJ34, as I mentioned, is very short; thus, we wanted to provide the mice with the highest possible levels of PJ34 during periods of stress. In some studies in larger animal models, PJ34 can be given as an intravenous drip. However, with a small 25-g mouse it is pretty difficult to do that, so we gave 2 intraperitoneal doses instead.

To answer to your second question about the 100% viability with the PJ34 at 48 hours, it is important to consider what kind of information is provided by the viability index, that is, the MTT or mitochondrial activity assay. Early decreases in the viability index in the presence of PJ34 may have actually represented mitochondrial stunning, rather than cell death. PJ34 is known to preserve myocardial high-energy phosphate levels during ischemia, thus our observation of full recovery of skeletal muscle at 48 hours is in line with previous studies in other tissues. In addition, tissue edema and skeletal muscle viability may not have a direct correlation in mice, as we believe it does in humans. Mice do not have fascia in their hind limbs, therefore the mice may tolerate severe edema without compromise to muscle viability. It is unclear that compartment syndrome that is observed in humans exist in mice. That may be an explanation why there is a discrepancy between viability and edema in these experiments.

Francis Moore, MD, Boston: I want to thank Dr Watkins and his group for starting to study this model that we have been involved with for quite a while. First, I have a comment. As far as we can tell, there are only 3 active cells in this injury: endothelium, the muscle, and the mast cells. In view of your $PARP^{-/-}$ knockout mouse, it would be my general advice that you should examine mast cells and see if there is any effect on mast cell degranulation, as we are relatively sure that there is a mast cell protease that is crucial to this injury.

Then the question I have is, with cell necrosis, do you know or do you have any way of knowing whether that is due to apoptosis or whether that is due to more conventional cell necrosis as you might see with complement or neutrophils.

Dr Hua: I do not know the specific answer to this question. The mechanism of PARP in apoptosis and its overactivation leading to cell death—I am not sure we can differentiate that. We do see histologic evidence of muscle fiber injury, but we are not yet able to differentiate between necrosis vs apoptosis. Presumably, cell death and tissue injury in this model is as a result of cell lysis due to the overwhelming energy crisis caused in part by overactivation of PARP.

Thomas Colacchio, Lebanon, NH: Just a follow-up question. Dr Moore suggested one potential mechanism but have you or your group begun to postulate the difference between the $PARP^{-/-}$ knockout strain and the PJ34 strain in terms of why there was an apparent enhancement of protection in the treated strain vs the $PARP^{-/-}$ knockout strain?

Dr. Hua: PJ34 has potent and specific activity against all 7 PARP isoforms. Thus, a single dose of water-soluble PJ34 will be distributed throughout the body, and will not have comparable temporal effects on the activity of a single PARP isoform as would be observed in the genetic PARP-1 knockout strain. Furthermore, the 30- to 60-minute half-life of PJ34 will also make it less effective than the genetic knockout.