Original Research

Pyruvate-enriched resuscitation: metabolic support of post-ischemic hindlimb muscle in hypovolemic goats

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Abstract

Tourniquet-imposed ischemia-reperfusion of extremities generates reactive oxygen and nitrogen species (RONS), which can disrupt intermediary metabolism and ATP production. This study tested the hypothesis that fluid resuscitation with pyruvate, a natural antioxidant and metabolic fuel, ameliorates the deleterious effects of ischemia-reperfusion on intermediary metabolism in skeletal muscle. Anesthetized male goats (~25 kg) were bled to a mean arterial pressure of 48 ± 1 mmHg and then subjected to 90 min hindlimb ischemia with a tourniquet and femoral crossclamp, followed by 4-h reperfusion. Lactated Ringers (LR) or pyruvate Ringers (PR) was infused intravenous for 90 min, from 30 min ischemia to 30 min reperfusion, to deliver 0.05 mmol kg^{-1} min^{-1} lactate or pyruvate. Time controls (TC) underwent neither hemorrhage nor hindlimb ischemia. Lipid peroxidation product 8-isoprostane, RONS-sensitive aconitase and creatine kinase activities, antioxidant superoxide dismutase activity, and phosphocreatine phosphorylation potential ([PCr]/[{Cr}{P_i}]), an index of tissue energy state, were measured in reperfused gastrocnemius at 90 min resuscitation ($n = 6$ all groups) and 3.5 h post-resuscitation ($n = 8$ TC, 9 LR, 10 PR). PR more effectively than LR suppressed 8-isoprostane formation, prevented inactivation of aconitase and creatine kinase, doubled superoxide dismutase activity, and augmented [PCr]/([Cr][P_i]). Pyruvate-enriched Ringer's is metabolically superior to Ringer's lactate for fluid resuscitation of tourniqueted muscle.

Keywords: Creatine kinase, hypovolemia, 8-isoprostane, phosphorylation potential

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Introduction

The application of tourniquets to minimize post-traumatic blood loss imposes ischemia on the wounded limb.[1,2] Upon tourniquet release, oxygenated blood is reintroduced into the reperfused tissue forming reactive oxygen and nitrogen species (RONS), which have been implicated in the pathogenesis of metabolic dysfunction.[1,3–5] RONS can inactivate enzymes catalyzing intermediary metabolism and high-energy phosphate transfer reactions, thereby compromising ATP supply for cellular functions.[4–8] Thus, treatments that protect these enzymes could preserve energy resources of reperfused muscle.

Lactated Ringer's is a mainstay among crystalloids given to trauma victims.[9] Because lactate is not an antioxidant, it cannot protect RONS-sensitive enzymes after ischemia-reperfusion.[9–11] Pyruvate provides both a metabolic fuel and an antioxidant.[3,10–12] Substituting pyruvate for lactate in Ringer's resuscitation would deliver an antioxidant capable of scavenging RONS and protecting enzymes, while supporting ATP production.[6,11,13]

This study tested two hypotheses: (1) substituting pyruvate-enriched Ringer's for lactated Ringer's could dampen RONS formation, protect key metabolic enzymes, and bolster the energy state of skeletal muscle exposed to ischemia-reperfusion in the setting of hypovolemia; (2) these beneficial effects persist for at least 3.5 h after terminating fluid resuscitation. Pyruvate's antioxidant and metabolic capabilities were examined in hindlimb muscle (gastrocnemius) subjected to tourniquet-imposed ischemia-reperfusion and harvested at 30 min reperfusion, i.e. 90 min fluid resuscitation, or at 4 h of reperfusion. It is shown that pyruvate-enriched resuscitation protected RONS-sensitive enzymes and activated the antioxidant enzyme superoxide dismutase (SOD) while bolstering tissue energy reserves more effectively than Ringer's lactate.

Materials and methods

Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center and adhered to the *Guide to the Care and Use of Laboratory Animals* (US National Research Council publication 85-23, revised 2011). Forty-five male Boer goats, 25–30 kg, were randomly assigned to the different treatments and protocols.

Surgical preparation

Goats were sedated by ketamine ($5\,mg\,kg^{-1}$)/midazolam ($0.2\,mg\,kg^{-1}$) cocktail injection into the left jugular vein under manual restraint.[14] After intubation, anesthesia was maintained by mechanical ventilation with 1–2% isoflurane supplemented with 100% O_2. After placing the goat in the left lateral decubitus position, the right jugular vein, carotid artery, and femoral vein were surgically exposed for placement of saline-filled cannulae. A pressure transducer (Argon Medical Devices, Athens, TX, USA) was connected to the carotid cannula to monitor arterial blood pressure. Arterial O_2 saturation was monitored by pulse oximetry. All goats were administered $650\,U\,kg^{-1}$ of heparin to fully anticoagulate the blood. Body temperature was monitored with an anal thermal probe and maintained at 38–39°C by placing the animals on water-circulating heating pads.

Hemorrhage, hindlimb ischemia, and fluid resuscitation

After surgical preparation was complete and baseline measurements were recorded, goats were exsanguinated by a controlled hemorrhage ($20\,mL\,min^{-1}$) from the jugular vein to lower the mean arterial pressure to 48 ± 1 mmHg. Hemorrhage volume was 220 ± 13 mL, i.e. $8.5 \pm 0.6\,mL\,kg^{-1}$. After arterial pressure stabilized at the target value, an atraumatic vasoclamp was applied to the right femoral artery and a veterinary tourniquet was tightened around the right hindlimb proximal to the femoral cutdown. Beginning 30 min after tourniquet placement, Ringer's solution enriched with lactate (LR) or pyruvate (PR) was infused for 90 min into the jugular vein with a Minipuls-2 roller pump (A-Vox Systems, San Antonio, TX, USA) at a rate sufficient to deliver four times the exsanguinated volume (total infused volume $35\,mL\,kg^{-1}$). The LR and PR solutions were prepared in double-distilled water within 30 min of infusion. Sodium salts of *L*-Lactate or pyruvate (Sigma-Aldrich, St. Louis, MO) were added at concentrations (110–130 mM) calibrated to deliver these compounds at a rate of $0.05\,mmol\,kg^{-1}\,min^{-1}$. NaCl was added to bring the total Na^+ concentration to 130 mEq/L; the media also contained 4 mM KCl and 1.5 mM $CaCl_2$. LR and PR were sterilized by filtration (0.22 um pore; Millipore Stericup®) and stored in autoclaved flasks before infusion.

At 60 min fluid resuscitation, the vasoclamp and tourniquet were released to abruptly reperfuse the hindlimb. Time control (TC) goats were surgically prepared, heparinized and instrumented, but were not subjected to hemorrhage, resuscitation, or hindlimb ischemia-reperfusion. Mean arterial pressures, heart rates, and electrocardiogram in these TC, LR, and PR experiments were reported recently.[15]

At the end of the experiment, biopsies of the right gastrocnemius were snap-frozen with liquid N_2 pre-cooled Wollenberger tongs, immersed in liquid N_2, and stored at −80°C. The LR- and PR-resuscitated goats were subjected to an abbreviated protocol, in which the gastrocnemius was harvested at 90 min of fluid resuscitation ($n = 6$ per group) or the full protocol in which the goats recovered for 4 h after hindlimb reperfusion before harvesting the gastrocnemius ($n = 9$ LR, 10 PR). Gastrocnemius was sampled after equivalent abbreviated ($n = 6$) and full ($n = 8$) TC protocols. Blood samples (*c.* 1.5 mL) were withdrawn from the carotid artery at predetermined time points and centrifuged; the plasma supernatant was flash-frozen and extracted.[11] Pyruvate and lactate in plasma extracts were measured by spectrophotometric assays.[16]

Gastrocnemius metabolites and enzymes

Frozen muscle biopsies were pulverized in liquid N_2-cooled porcelain mortars. Metabolites, including ATP, phosphocreatine (PCr), creatine (Cr), inorganic phosphate (P_i), lactate, pyruvate, and citrate, were extracted from the pulverized muscle[11] and assayed spectrophotometrically.[16] Phosphocreatine phosphorylation potential, i.e. [PCr]/([Cr][P_i]), provided a measure of the muscle's free energy of ATP hydrolysis.[11,13]

Proteins were extracted from the pulverized muscle in a phosphate buffer.[17] Total protein concentrations in the extracts were determined colorimetrically using a Coomassie Plus Bradford Kit (Pierce, Rockford, IL, USA).[18] Activities of RONS-sensitive (creatine kinase, aconitase) and RONS-inert (lactate dehydrogenase) enzymes in these extracts were measured by spectrophotometric assays[6,16] and expressed as IU/mg protein. SOD activity was measured with an analytical kit (no. 706002, Cayman Chemical, Ann Arbor, MI, USA).

Gastrocnemius 8-isoprostane content

8-isoprostane, a product of RONS-mediated peroxidation of arachidonic acid, provided a marker of oxidative stress.[19,20] 8-isoprostane was extracted from pulverized muscle and spectrophotometrically measured at 412 nm in a 96-well plate reader (BioTek KCJunior, Winooski, VT, USA) using an enzyme-linked immunoassay kit (no. 516351, Cayman Chemical). Values in the LR, PR, and full TC protocol groups are normalized to the mean value of the abbreviated TC protocol.

Statistical analysis

Mean values ± SEM are reported. Plasma lactate and pyruvate concentrations among the LR, PR, and TC groups were compared by two factor (treatment, time) analyses of variance (ANOVA). Other variables were compared among treatments by single-factor ANOVA. When statistically significant treatment effects were detected, Student-Newman Keuls *post hoc* tests were applied to identify the specific between-group differences. Statistical analyses were

performed using SigmaStat v10. Values of $P < 0.05$ were considered statistically significant.

Results

Arterial plasma pyruvate and lactate concentrations

Pyruvate Ringer's administration increased plasma pyruvate concentration (Figure 1(a)) from 0.17 ± 0.02 to 2.5 ± 0.3 mM, a 4.5-fold increase over lactated Ringer's (LR; $P < 0.001$) and 16 fold above the TC value ($P < 0.001$). Within 30 min after PR infusion, plasma pyruvate fell to concentrations similar to those in the LR experiments. During resuscitation, LR increased plasma lactate concentration ninefold ($P < 0.001$ vs. TC) to 9.9 ± 0.9 mM (Figure 1(b)). PR also produced a marked increase in plasma lactate, from 0.7 ± 0.1 to 5.0 ± 0.6 mM ($P < 0.001$ vs. TC). At 3.5-h post-resuscitation, plasma lactate concentration in the LR group (3.4 ± 1.0 mM) remained 1.8- and 3.4-fold above the respective PR and TC values of 1.9 ± 0.2 ($P = 0.03$) and 1.0 ± 0.2 mM ($P = 0.01$).

Muscle pyruvate, lactate, and citrate contents

Lactate dehydrogenase catalyzes a redox-sensitive equilibrium between pyruvate and lactate in the cytosol. In the mitochondrial matrix, pyruvate carboxylation generates Krebs cycle intermediates and, thus, increases citrate content.[3,21–23] Accordingly, to assess these cytosolic and mitochondrial processes of pyruvate metabolism, pyruvate, lactate, and citrate contents were measured in muscle reperfused for 30 min (i.e. at 90-min fluid resuscitation) and 4 h (3.5-h post-resuscitation). As expected, muscle pyruvate content (Figure 2(a)) was higher during resuscitation with PR than during LR resuscitation or TC ($P < 0.001$). Notably, pyruvate content was still be elevated 3.5 h after PR resuscitation. Muscle lactate contents were increased to similar extents by LR and PR resuscitation ($P < 0.001$ vs. TC), indicating substantial conversion of pyruvate to lactate in PR-resuscitated muscle. Lactate contents did not differ among the three groups 3.5 h after resuscitation (Figure 2(b)).

Muscle citrate content (Figure 2(c)) paralleled pyruvate content. Thus, PR resuscitation increased muscle citrate content by nearly threefold vs. TC ($P < 0.001$) and by 65% vs. LR ($P = 0.001$). Resuscitation with LR increased muscle citrate content by 67% vs. TC ($P = 0.03$). Citrate content fell by 50% over the 3.5 h following PR resuscitation, but remained above the respective contents in the LR ($P = 0.04$) and TC ($P = 0.002$) groups.

8-Isoprostane

The lipid peroxide 8-isoprostane is an indicator of oxidative stress and antioxidant deficiency in tissues. The combination of hemorrhage, LR resuscitation, and hindlimb ischemia-reperfusion subjected the gastrocnemius to appreciable oxidative stress: 8-isoprostane content, measured 30 min after reperfusion, increased fourfold vs. the TC value ($P = 0.015$; Figure 3). In contrast, resuscitation with PR blunted oxidative stress: 8-isoprostane content in this group was 28% of the LR value ($P = 0.007$) and did not differ from the TC value.

Muscle 8-isoprostane content fell over the 3.5 h following LR resuscitation (Figure 3) but remained 49% above the TC value ($P = 0.006$). 8-isoprostane content 3.5 h after PR resuscitation was 31% below that of the LR group ($P = 0.035$) and remained similar to the TC value. Thus, ischemia-reperfusion of the gastrocnemius following hemorrhage in combination with LR resuscitation imposed oxidative stress which was mitigated by PR resuscitation.

Activities of oxidant-sensitive and antioxidant enzymes

Activities of two oxidant-sensitive metabolic enzymes, the energy-shuttling enzyme creatine kinase (Figure 4(a)) and the Krebs cycle enzyme aconitase (Figure 4(b)), and lactate dehydrogenase (Figure 4(c)), an enzyme more resistant to oxidative stress, were measured in gastrocnemius reperfused for 30 min and 4 h. At 30 min reperfusion, i.e. 90 min of fluid resuscitation, and 3.5 h later, creatine kinase and aconitase activities in the LR group were only about half those in the TC muscle. In contrast, PR resuscitation preserved activities of creatine kinase and aconitase, both during and 3.5 h after fluid resuscitation. Thus, resuscitation with PR but not LR preserved activities of oxidant-sensitive creatine kinase and aconitase in skeletal muscle subjected to ischemia-reperfusion and the attendant oxidative stress. In contrast, lactate dehydrogenase activities did not differ among the TC, LR-resuscitated and PR-resuscitated muscles and remained stable over the post-resuscitation period (Figure 4(c)).

SOD is a pivotal antioxidant enzyme that reduces the free radical superoxide to hydrogen peroxide. SOD activity did not differ in LR-resuscitated vs. TC muscle at 4 h reperfusion (Figure 5). On the other hand, PR resuscitation doubled SOD activity vs. LR ($P = 0.027$) and TC ($P = 0.01$).

Tissue energy metabolites

Resuscitation with PR appreciably lowered [P_i] vs. LR and TC, and this effect persisted 3.5 h after PR administration (Figure 6). PR tended to increase ATP and PCr contents and decrease Cr content, although these effects were not statistically significant. PR increased [PCr]/([Cr][P_i]), a measure of ATP phosphorylation potential and, thus, tissue energy state,[13,24] vs. LR and TC ($P < 0.001$; Figure 7). This effect persisted after resuscitation; 3.5 h later, [PCr]/([Cr][P_i]) in post-PR muscle remained elevated vs. post-LR ($P < 0.001$) and TC ($P = 0.003$) muscles. Thus, resuscitation with pyruvate-enriched Ringer's solution augmented the post-ischemic muscle's energy state more effectively than LR, and this effect persisted for 3.5 h after resuscitation.

Discussion

This study demonstrated that resuscitation with a pyruvate-fortified Ringer's solution during hypovolemia and hindlimb ischemia-reperfusion dampens oxidative stress, preserves oxidant-sensitive metabolic enzymes, and bolsters phosphorylation potential in hindlimb muscle, more effectively than lactate-enriched Ringer's, both during fluid resuscitation and after pyruvate cleared

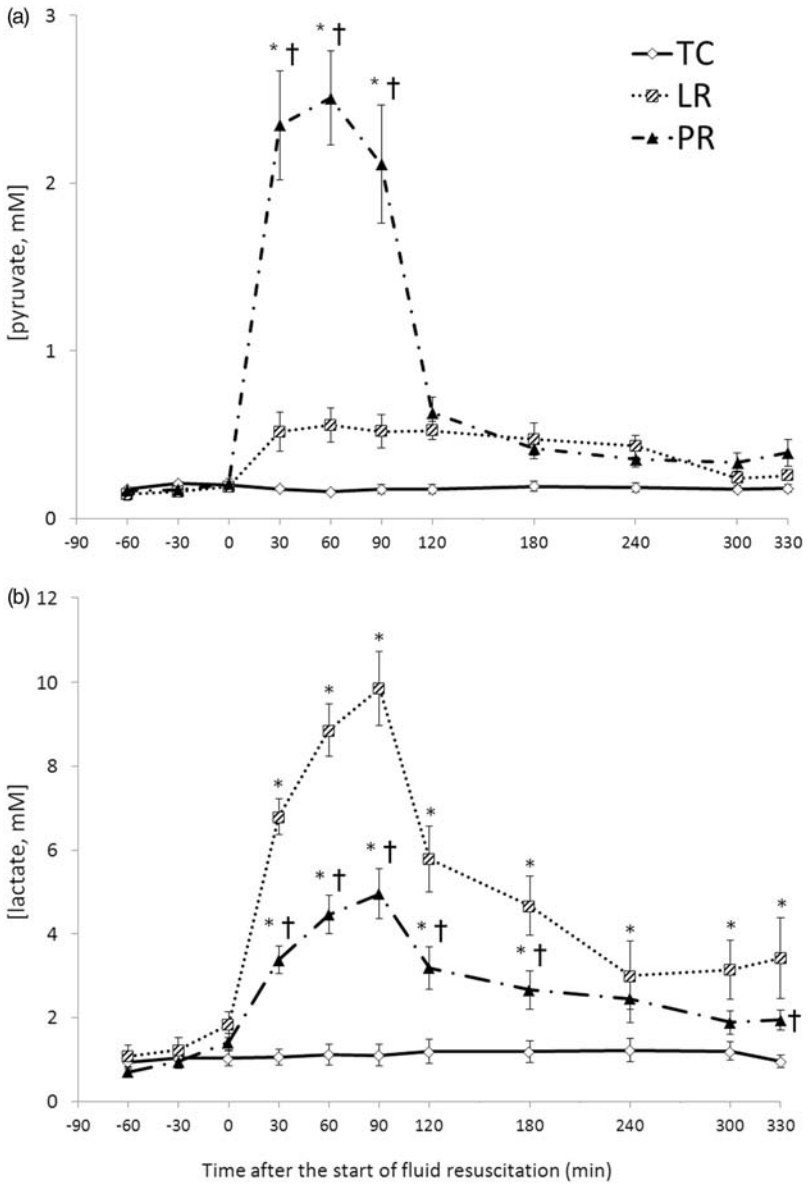

Figure 1 Effects of resuscitation with lactate- and pyruvate-enriched Ringer's on plasma pyruvate (a) and lactate (b) concentrations. Values are mean ± SEM. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

from the circulation. Compared to LR, resuscitation with PR suppressed 8-isoprostane formation, activated the antioxidant enzyme SOD, protected RONS-sensitive creatine kinase and aconitase, and augmented phosphocreatine phosphorylation potential in gastrocnemius subjected to ischemia-reperfusion superimposed on hypovolemia. These favorable effects persisted 3.5 h after PR administration, i.e. 4 h after hindlimb reperfusion. In clinical settings, the persistence of PR's effects could afford extended protection of the wounded extremity during transport to the trauma center and surgical repair. To our knowledge, this study is the first to examine pyruvate's effect on skeletal muscle energy state in animals subjected to hypovolemia, hindlimb ischemia-reperfusion, and fluid resuscitation.

Antioxidant effects of pyruvate

Reactive oxygen and nitrogen species are generated upon reperfusion, when oxygen is reintroduced into previously ischemic tissue.[1,25] In this study, 8-isoprostane, a product of oxidative stress, increased fourfold in reperfused muscle resuscitated with LR. In contrast, PR resuscitation blunted the formation of 8-isoprostane upon hindlimb reperfusion and this antioxidant effect persisted at least another 3.5 h. In a swine model of irreversible hemorrhagic shock, sodium pyruvate delayed cardiovascular decompensation by 70–80 min vs. NaCl and stabilized hepatic antioxidant defenses.[26,27] In addition, goats resuscitated with lactated Ringer's during hypovolemia and hindlimb ischemia-reperfusion had higher muscle nitrotyrosine content, a footprint of nitrosative stress, than goats resuscitated with

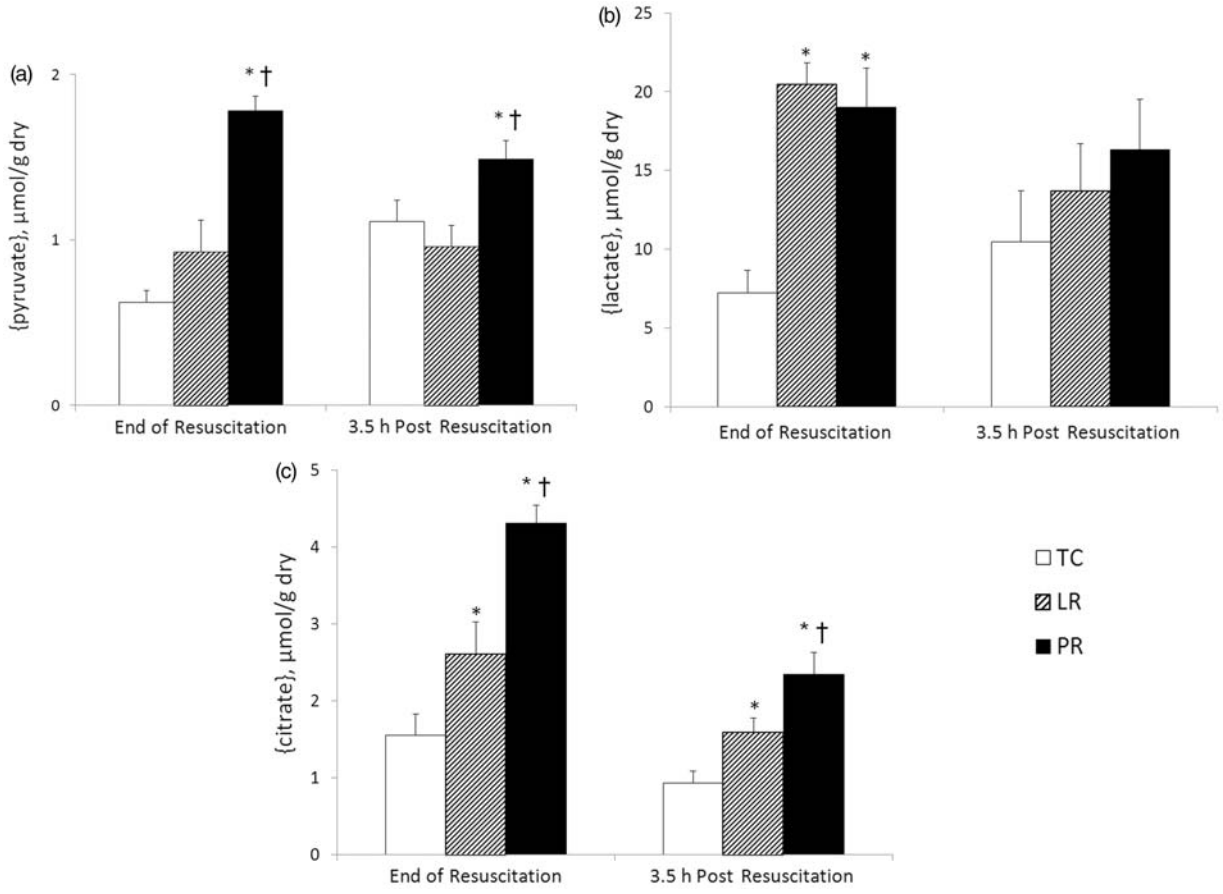

Figure 2 Pyruvate (a), lactate (b), and citrate (c) contents in post-ischemic gastrocnemius. Metabolites were measured in muscle biopsied at 30 min or 4 h hindlimb reperfusion or at the corresponding TC time points. In this and the following figures, $n = 6$ per group at end of fluid resuscitation, and $n = 8$, 9, and 10, respectively, for TC, LR, and PR at 3.5 h post-fluid resuscitation. Values are mean ± SEM. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

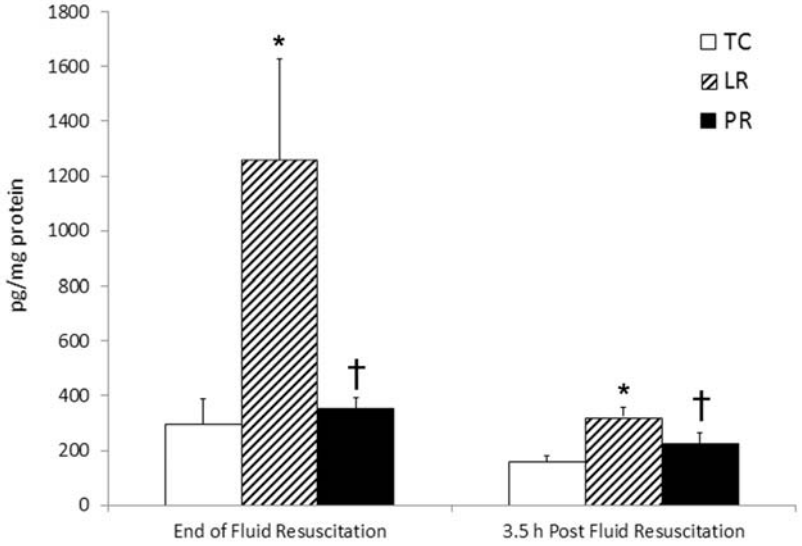

Figure 3 8-isoprostane content in post-ischemic gastrocnemius. 8-isoprostane was measured in gastrocnemius at the end of fluid resuscitation and at 3.5 h post-resuscitation, i.e. 4 h hindlimb reperfusion. Values are mean ± SEM. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

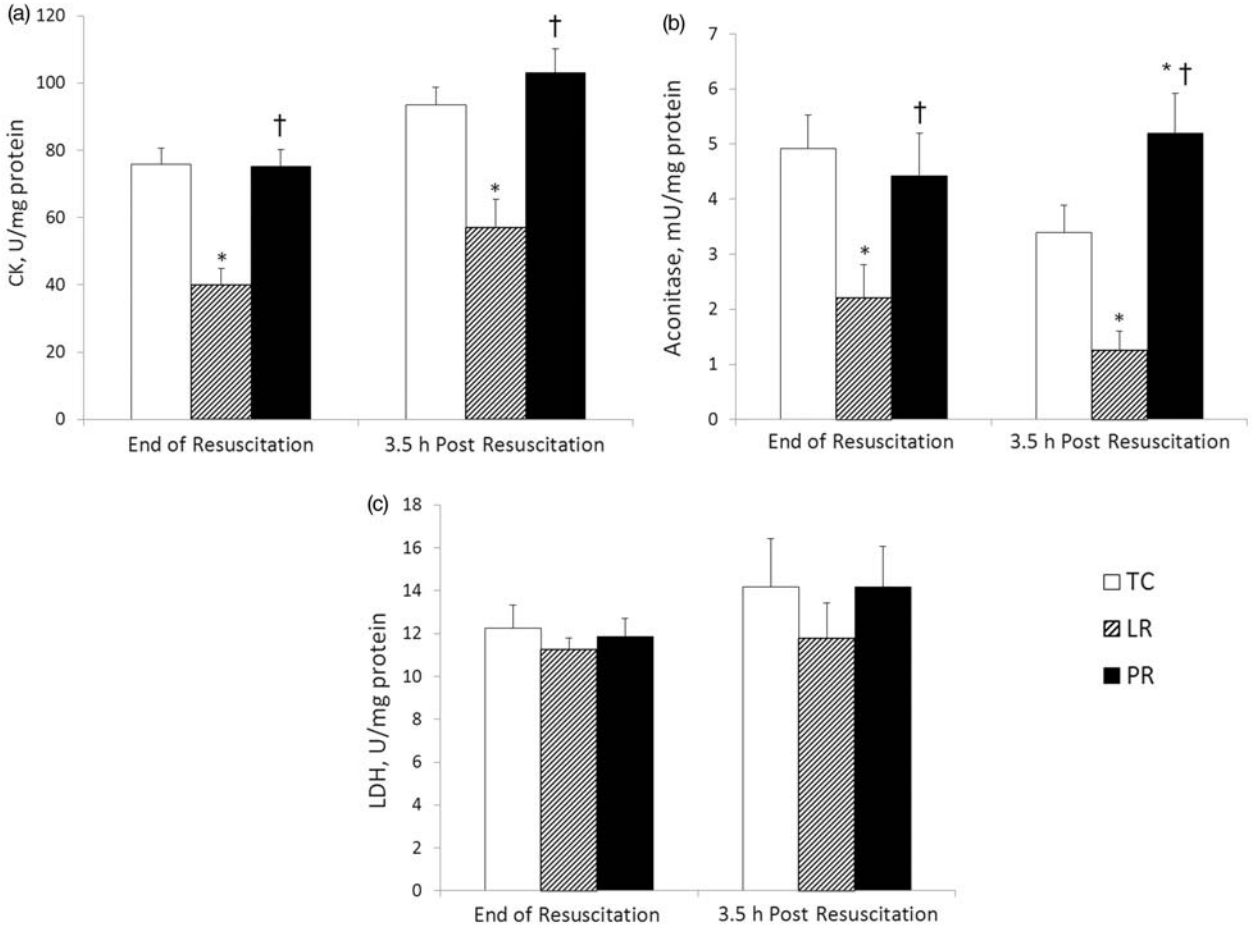

Figure 4 Creatine kinase (a), aconitase (b), and lactate dehydrogenase (c) activities in gastrocnemius. Enzyme activities (mean ± SEM), measured at 90 min fluid resuscitation and at 3.5 h post-resuscitation, are normalized to total protein contents measured in the same extracts. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

pyruvate-enriched Ringer's solution.[6] Thus, pyruvate's antioxidant capabilities enabled PR to scavenge RONS more effectively than LR.

Pyruvate protection of oxidant-sensitive enzymes

Ischemia-reperfusion can compromise intermediary metabolism and ATP production. Several key enzymes, including those involved in energy production and shuttling, are RONS targets.[4–6,28,29] In this study, pyruvate's antioxidant properties were exploited to scavenge RONS and thereby protect oxidant-sensitive creatine kinase and aconitase in muscle subjected to ischemia-reperfusion. Creatine kinase and aconitase activities fell sharply during ischemia-reperfusion and LR resuscitation, and remained depressed for 4 h after hindlimb reperfusion. However, muscle pyruvate content remained elevated at 4 h reperfusion, i.e. 3.5 h after PR administration, and, thus, PR protection of aconitase and creatine kinase persisted. Lactate has proven ineffective at scavenging the reactive oxygen and nitrogen species capable of inactivating enzymes.[6,11]

A cytosolic enzyme, lactate dehydrogenase, is released from myocytes damaged by ischemia and reperfusion, and loss of lactate dehydrogenase activity is taken to indicate disruption of the plasma membrane.[30] The similarity of muscle lactate dehydrogenase activities in the LR, PR, and TC groups argues that the inactivation of creatine kinase and aconitase in the LR-resuscitated muscle was not due to cellular damage and enzyme leakage but rather RONS-induced enzyme inactivation. Thus, hindlimb ischemia-reperfusion superimposed on central hypovolemia can generate RONS capable of inactivating oxidant-sensitive enzymes. Resuscitation with PR not only effectively scavenged RONS in the gastrocnemius but also protected these important enzymes of intermediary metabolism.

SOD, a crucial enzymatic component of the muscle's antioxidant armamentarium, catalyzes a single electron reduction converting the free radical superoxide to the non-radical hydrogen peroxide, thereby initiating the detoxification of RONS in tissues. The doubling of SOD activity effected by PR in the post-ischemic muscle may contribute to the protection of RONS-sensitive enzymes. Although it is not yet clear how pyruvate might activate SOD, recent findings in muscle preparations suggest a possible mechanism. In skeletal myocytes freshly isolated from goat *latissimus dorsi*, exposure to hydrogen peroxide decreased activity and content of CuZn SOD, but the addition of antioxidant tea catechins increased the activity and content of the enzyme to levels even above those of

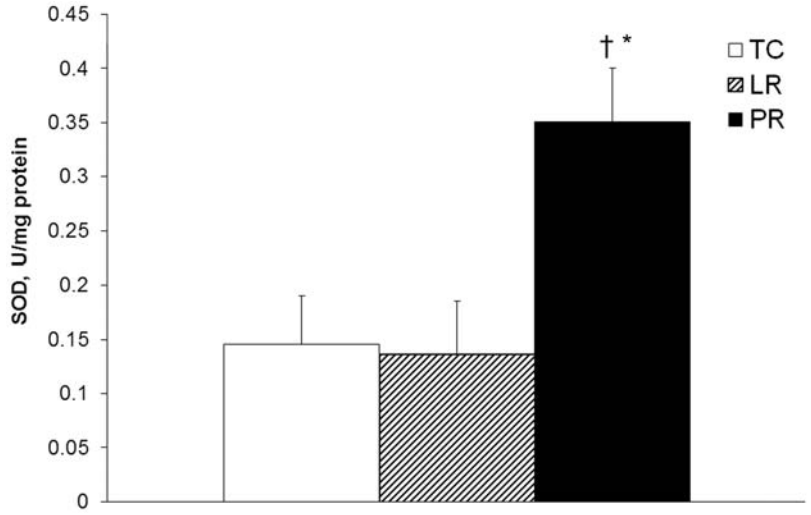

Figure 5 Superoxide dismutase activity in post-ischemic muscle. Total superoxide dismutase activity (mean ± SEM) was measured in protein extracts of gastrocnemius biopsied at 4 h reperfusion in 9 LR and 10 PR experiments, and in 8 TC experiments. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

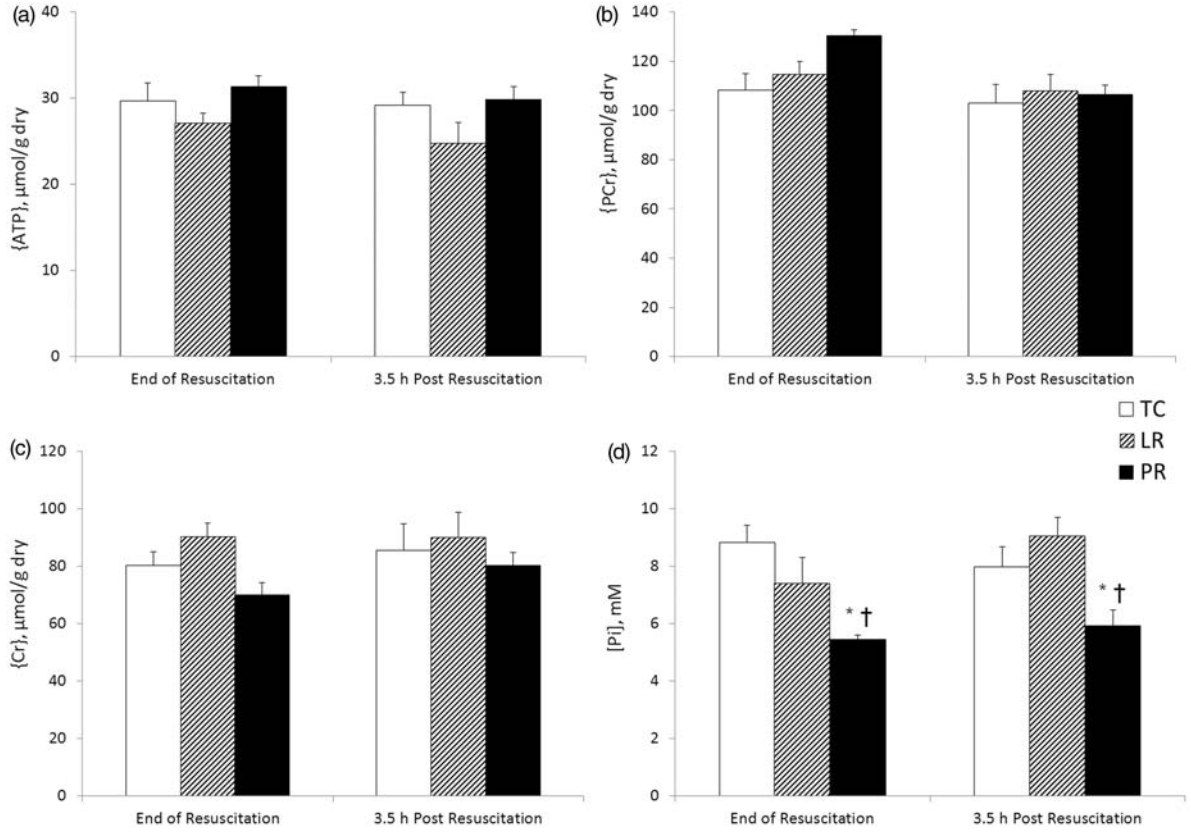

Figure 6 Energy metabolites in post-ischemic gastrocnemius. Adenosine triphosphate {ATP}, phosphocreatine {PCr}, and creatine {Cr} contents and intracellular inorganic phosphate [P_i] concentrations in gastrocnemius were measured at 90 min fluid resuscitation or at 3.5 h post-resuscitation in LR- and PR-resuscitated goats or in the corresponding TC groups. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. Values are mean ± SEM. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

hydrogen peroxide-free control cells.[31] Pyruvate scavenges peroxides in direct, non-enzymatic chemical reactions,[3,12] and this mechanism arguably may have prevented SOD inactivation. Indeed, in myocardium of Wistar rats, overexposure to isoproterenol decreased total SOD activity by 60%, but orally administered pyruvate dose-dependently maintained SOD activity in the face of isoproterenol excess.[32]

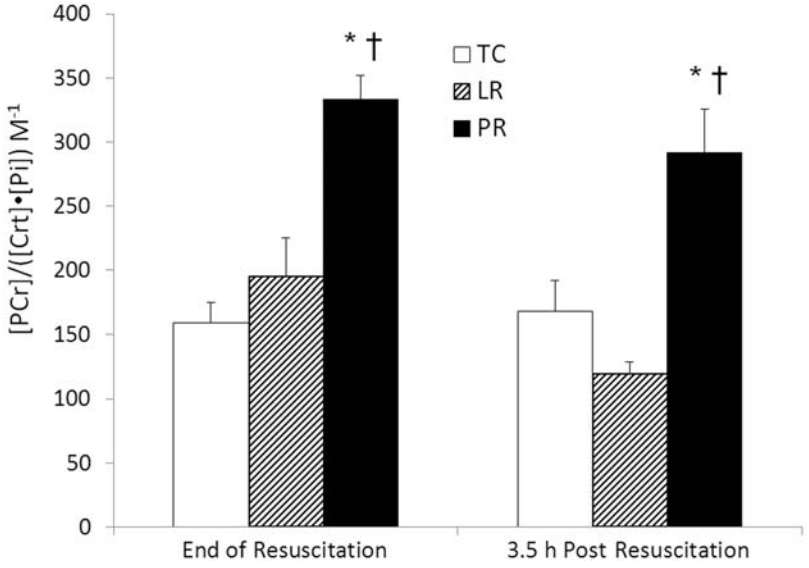

Figure 7 Effect of lactate- vs. pyruvate-enriched fluid resuscitation on phosphorylation potentials of post-ischemic gastrocnemius. Phosphocreatine phosphorylation potentials ([PCr]/([Cr][P_i])) were computed from phosphocreatine [PCr], creatine [Cr], and inorganic phosphate [P_i] concentrations measured at 90 min fluid resuscitation or at 3.5 h post-resuscitation in the LR- and PR-resuscitated goats, or in the corresponding TC groups. *$P < 0.05$ vs. TC; †$P < 0.05$ vs. LR. Values are mean ± SEM. TC: sham time control; LR: lactate Ringer's resuscitation; PR: pyruvate Ringer's resuscitation

Anaplerosis and citrate content

Pyruvate-enriched Ringer's resuscitation increased muscle citrate content more than LR, and this effect was still evident 3.5 h after resuscitation. Anaplerotic pyruvate carboxylation generating malate and oxaloacetate increases other Krebs cycle intermediates including citrate.[3,21–23] Citrate both prevents aconitase inactivation by peroxynitrite and contributes to aconitase reactivation following oxidative stress.[29,33] In addition, citrate concentration-dependently dampens aconitase inactivation by binding to the enzyme's catalytic site.[33] Moreover, after inactivation of aconitase by RONS, citrate promotes the reinsertion of iron into the cubane iron-sulfur cluster at aconitase's catalytic core, thereby reactivating the enzyme.[29] An increase in citrate content can also bolster cellular antioxidant defenses by diverting glycolytic flux into the NADPH-generating hexose monophosphate pathway.[3,34] As noted below, oxidative decarboxylation of the Krebs cycle intermediate malate by $NADP^+$-dependent malate decarboxylase also generates NADPH.

Three mechanisms may have contributed to the persistent elevation of muscle pyruvate content 3.5 h after PR resuscitation. First, plasma pyruvate concentration at this time, 0.39 ± 0.08 mM, remained above the baseline concentration of 0.17 ± 0.02 mM and approached plasma pyruvate concentrations (*c.* 0.5 mM) at which antioxidant effects were detected in hypovolemic goats.[35] Second, citrate, which remained elevated 3.5 h post-PR resuscitation, may serve as a source of pyruvate via the sequential actions of three enzymes active in skeletal muscle: ATP citrate lyase, malate dehydrogenase, and $NADP^+$-dependent malate decarboxylase.[36–38] The latter enzyme converts malate to pyruvate with concomitant production of NADPH. Thirdly, conversion of lactate to pyruvate has been demonstrated in mitochondrial matrix of exercising skeletal muscle[39]; conceivably, such conversion may occur in the post-ischemic muscle.

Pyruvate enhancement of tissue energy state

The loss of central blood volume during hemorrhage compromises end-organ perfusion and, thus, delivery of oxygen and nutrients necessary for cellular energy production. This effect is compounded in the wounded extremity by ischemia-reperfusion imposed by tourniquet application and release.[7] In this study, PR resuscitation during hypovolemia and hindlimb ischemia-reperfusion augmented muscle phosphorylation potential vs. TC and LR, an effect that persisted 3.5 h after PR resuscitation. Ringer's solution containing the pyruvate derivative ethyl pyruvate augmented hindlimb ATP content more effectively than LR in mice subjected to unilateral hindlimb ischemia-reperfusion.[40] Thus, pyruvate-enriched fluids have been found to augment tissue energetics during metabolic stress in a manner superior to fluids containing lactate.

Pyruvate may augment the muscle energy state by several mechanisms, including direct supply of substrate, formation of Krebs cycle intermediates, enhancement of glycolytic flux secondary to cytosolic NADH oxidation, and antioxidant protection of the energy-generating metabolic machinery.[3,10,13] In this study, resuscitation with PR decreased tissue Pi concentration, which contributed to the enhancement of PCr phosphorylation potential, [PCr]/([Cr][P_i]). In swine, cardiac arrest with pyruvate-enriched cardioplegia preserved phosphorylation potential more effectively than lactate-based cardioplegia, attributable to the divergent effects of these solutions on intracellular (P_i).[11] That study examined myocardium sampled 3 min after reperfusion, while in the present study, PR augmentation of energy resources of post-ischemic skeletal

muscle was detected at 90 min PR infusion and also a full 3.5 h later, in a manner superior to LR.

Limitations

Pyruvate Ringer's resuscitation increased peak plasma pyruvate concentrations to 2.5 ± 0.3 mM, somewhat below the optimally cytoprotective range of 5–10 mM.[6,13,26,27] Because pyruvate was administered as a sodium salt, its dosage was calibrated to achieve metabolic efficacy while avoiding hypernatremia. The severity of hemorrhage in this study was moderate; it is unclear if pyruvate resuscitation would be effective against more profound hypovolemia and hypotension. Circulating lactate dehydrogenase interconverts pyruvate and lactate with an equilibrium favoring lactate formation, such that PR resuscitation sharply increased plasma lactate, while LR only moderately increased plasma pyruvate concentration (Figure 1). These changes in plasma composition may have narrowed the differences in the effects of the two Ringer's solutions.

There are two prominent SOD isoenzymes in skeletal muscle, extracellular CuZn-dependent SOD and mitochondrial Mn-dependent SOD. Although total SOD was increased by PR resuscitation, the impact of PR on the individual isoenzymes is not known.

This study examined the acute effects of PR vs. LR on muscle biochemistry within the first few hours of hindlimb reperfusion, under continuous anesthesia. The long-term effects of these treatments on muscle ultrastructure and contractile function, particularly over the days following the acute insult, have not been examined. Finally, the effects of pyruvate-enriched colloid formulations and anti-inflammatory pyruvate derivatives such as ethyl pyruvate[40] merit investigation.

Conclusions

Pyruvate-enriched fluid resuscitation effectively scavenged RONS in skeletal muscle subjected to ischemia-reperfusion superimposed on hypovolemia, more effectively than LR resuscitation. Consequently, pyruvate protected the activities of enzymes catalyzing ATP production and shuttling, and augmented muscle phosphorylation potential. Importantly, these favorable effects of pyruvate persisted at least 3.5 h after resuscitation. Ringer's lactate did not produce these antioxidant, metabolic, and energetic benefits. These findings may help stimulate the formulation of crystalloids for more effective management of hypovolemia and ischemia-reperfusion stress in trauma victims.

Author Contributions: HAG performed surgical preparation, conducted experiments, conducted analytical measurements of enzymes and metabolites, performed data reduction, analysis and statistics, prepared figures and drafted manuscript. DWW and AGW assisted with surgical preparation, co-conducted experiments, collected samples for analysis and assisted with analytical measurements. BH assisted with experimental design, performed surgical preparation, co-conducted experiments, and assisted with sample collection. JS prepared resuscitative fluids, assisted with sample collection, analytical measurements, data reduction and analysis, and trained HAG and DWW in metabolite and enzyme assays. JPH and DRS assisted with surgical preparation and sample collection and co-conducted experiments and serum analyses. AHOY co-designed experiments, assisted with surgical preparation, and assisted with data analysis and interpretation. RTM co-designed experiments, guided data analysis, interpretation and manuscript preparation, and edited the manuscript. All authors have approved the final manuscript.

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