Pyruvate Modulates Hepatic Mitochondrial Functions and Reduces Apoptosis Indicators during Hemorrhagic Shock in Rats

Pushpa Sharma, Ph.D.,* Kane T. Walsh, M.D.,† Kimberly A. Kerr-Knott, M.D.,† John E. Karaian, B.S.,‡ Paul D. Mongan, M.D.§

Background: Dysfunctional mitochondria have been widely accepted as one of the key targets and a mediator of secondary cell injury and organ failure during hemorrhagic shock (HS). The liver is known to be the first organ to display the signs of injury during HS. This report describes experiments to determine whether modulation of hepatic mitochondrial dysfunction by pharmacologic agents could prevent liver injury in rats subjected to HS.

Methods: In this study, Sprague-Dawley rats were either treated as controls or subjected to computer-controlled arterial hemorrhage (40 mmHg) for 60 min followed by resuscitation with hypertonic saline, hypertonic β-hydroxybutyrate, or hypertonic sodium pyruvate for the next 60 min before death. During the course of the experiment, animals were continuously monitored for hemodynamic and metabolic parameters. At the end of the experiment, the liver was excised and examined for oxidative injury, mitochondrial functions, expression of nitric oxide synthase, and indicators of apoptosis.

Results: In comparison to hypertonic saline and hypertonic β-hydroxybutyrate, pyruvate significantly protected the liver from oxidative injury, prevented the up-regulation of nitric oxide synthase, inhibited pyruvate dehydrogenase deactivation, and improved cellular energy charge and mitochondrial functions. In addition, pyruvate also reduced cleavage of poly(adenosine diphosphate ribose polymerase by preventing leakage of mitochondrial cytochrome c in the liver of HS animals.

Conclusions: These data suggest that modulation of mitochondrial metabolic functions is likely to be one of the important mechanisms by which pyruvate exerts its protective effects on the liver during HS and resuscitation in rats.

MULTIPLE organ failure in hemorrhagic shock (HS) has been commonly related to metabolic failure due to dysfunctional mitochondria. The liver is known to be the first organ to display the signs of injury during HS due to its high mitochondrial content, increased metabolic rate, lactic acid clearance, and increased production of oxygen and nitrogen species in comparison with other organs.1–3 A relation between mitochondrial dysfunction and the irreversibility of pathologic damage in liver has been reported.1–6 Therefore, it is logical to hypothesize that the pharmacologic agents that can reduce reactive oxygen and nitrogen species and prevent mitochondrial damage may offer a promising treatment for HS.

To test our hypothesis, we examined and compared the effects of pyruvate with another pharmacologic agent, β-hydroxybutyrate (β-HOB), in resuscitative fluids of rats subjected to HS. The rationale for the use of pyruvate and β-HOB is that they are antioxidants, decrease membrane permeability, and produce acetyl coenzyme A as the immediate substrate of the Krebs cycle to generate mitochondrial matrix reduced adenine dinucleotide for oxidative phosphorylation.7 The uptake of β-HOB by mitochondria is adenosine triphosphate (ATP) dependent, whereas pyruvate requires the activity of the mitochondrial enzyme pyruvate dehydrogenase, which is regulated by the content of its E-1α subunit.8 However, it is not known whether pharmacologic activation of pyruvate dehydrogenase complex (PDH) and up-regulation of its E-1α subunit could be linked to the improved hepatocellular ATP, mitochondrial functions, and decreased apoptosis. In addition, the use of β-HOB in the treatment of HS has not been extensively studied in HS. On the other hand, recent work from our laboratory and those of others have shown the use of pyruvate administration in restoring the cellular metabolic functions in the brain and heart and also in enhancing the survival rates during HS or ischemia by preventing the loss of ATP.7,9–12 Despite this, the mechanisms for these responses are not fully understood, and the role of pyruvate in restoring the mitochondrial energetic functions in one of the most important organs, the liver, is still unresolved. The use of pyruvate and β-HOB as alternate adjuvants to metabolic resuscitative fluids may therefore provide a rational approach for the development of an effective metabolic therapy for HS by maintaining the hepatocellular functions.

Materials and Methods

The protocol and experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Uniformed Services University of the Health Sciences at Bethesda, Maryland, with adherence to the Guide for Care and Use of Laboratory Animals.13

Animal and Surgical Preparation

Male Sprague-Dawley rats (Taconic Farms, German Town, NY) weighing 225–250 g were used in this study.
Rats were not fasted at the time of experiment. Animals were anesthetized by spontaneously breathing 1.6% halothane in room air through a nose cone. The femoral artery and vein were surgically cannulated with PE-50 polyethylene tubing for fluid administration, blood sampling, and continuous monitoring of blood pressure. The incision was closed with interrupted sutures. Core temperature (rectal) was maintained at 37°C ± 0.2°C with a heat lamp. The rats were continuously monitored for mean arterial blood pressure (MAP) by connecting the arterial catheter to a pressure transducer and computerized physiograph (LABVIEW 5; National Instruments, Austin, TX). The readings were recorded every 10 s and averaged per minute over the course of the experiment. The baseline MAP was stabilized by infusing normal saline. At this point, the concentration of halothane was reduced to 1% throughout the experiment.

Experimental Protocol

Induction of HS. Computer-controlled arterial hemorrhage to a target of MAP of 40 mmHg was achieved over 15 min by the withdrawal of blood from femoral arterial catheter using an Instech P720 peristaltic pump (Instech Laboratories Inc., Plymouth Meeting, PA). This MAP of 40 mmHg was maintained by either removal or infusion of blood for a 60-min (T60) shock period.

Resuscitation Groups. The animals were randomly assigned to the four treatment groups (n = 6 animals/group). In group 1, instrumented time control rats were anesthetized and instrumented in the same manner as rats in the other group but did not undergo arterial hemorrhage except the withdrawal of blood for laboratory investigation. Vital signs were recorded for 120 min by infusing normal saline three times the volume of blood lost. Groups 2, 3, and 4 were subjected to hemorrhage except the withdrawal of blood for laboratory investigation. The duration from harvesting of liver to homogenization was kept as short as possible (< 5 min). Tissue was minced, washed, and homogenized in ice-cold mitochondrial isolation buffer consisting of 0.25 m sucrose, 10 mM HEPES buffer, pH 7.4, and 1 mM EGTA in a ratio of 1:10 (vol/vol). Mitochondria were isolated by differential centrifugation at −4°C. In brief, cell membranes and nuclei were separated by centrifugation of tissue homogenate at 1,000g. The first supernatant was centrifuged at 10,000g for 15 min to get a mitochondrial pellet (S1). The supernatant thus obtained was centrifuged at high speed, 100,000g, for 60 min in a Beckman Ultra-centrifuge-TL100 (Scientific Surplus, LLC, Hills Borough, NJ) to obtain the cytosolic pellet. The mitochondrial pellet was washed three times in mitochondrial isolation buffer without EGTA to obtain relatively pure mitochondria for determining the respiratory rates, activation state of PDH, level of its E-1α subunit, and citrate synthase activities. The cytosolic fraction was used for Western analysis of cytochrome c. All measurements were performed in duplicate, and the average of the two from each of the six animals in each group was used for data analysis.

Analytical Procedures

Measurement of Liver Enzymes. The serum contents of liver enzymes alanine aminotransferase and aspartate aminotransferase were determined in our clinical chemistry laboratory at the Uniformed Services University of the Health Sciences.

Measurement of Tissue Lipid Peroxidation Level. Malondialdehyde, a degradation product of peroxidized phospholipids, was quantified as an indirect marker of oxidative stress using a colorimetric thiobarbituric acid assay kit from Calbiochem (La Jolla, CA). The Malondialdehyde concentrations were normalized to protein content and expressed as nmol/mg tissue protein.

Protein Assay. Protein contents in tissue, mitochondrial, or cytosolic samples were determined according to Bradford assay using bovine serum albumin as a standard.14

Determination of Adenine Nucleotides and Energy Charge in Liver. To determine the contents of cellular nucleotides, pulverized frozen T120 liver tissue was deproteinated by incubation in 1.5 M perchloric acid (3 ml/mg protein) for 10 min and then neutralized with 2 M KOH. Nucleotides ATP, ADP, and adenosine monophosphate (AMP) were determined as a measure of reduced adenine dinucleotide formation at 340 nm using hexokinase and glucose 6-phosphate dehydrogenase as
substrates. Energy charge was calculated as an indicator of cellular metabolic status in liver by using the equation:

\[
\text{Energy Charge} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{([\text{ATP}] + [\text{ADP}] + [\text{AMP}])}
\]

**Determination of Pyruvate Dehydrogenase Complex Activity.** The total PDH (PDHt) and the native PDH (PDHn) in their activated or dephosphorylated form at T120 were determined in mitochondrial samples. For the measurement of PDHn activity, the inactive (phosphorylated) form of the enzyme was converted to the “active” (dephosphorylated) form by incubating 25 mg mitochondrial protein in 1.0 ml of 10 mM MgCl2 and 10 mM CaCl2 for 5 min at 25°C. The activities of PDHn and PDHt were determined spectrophotometrically after reduction of \(p\)-iodonitrotetrazolium violet by PDH-dependent reduced adenine dinucleotide production at 500 nm. The PDH activity was calculated using the molar extinction coefficient of \(p\)-iodonitrotetrazolium violet (5.4 \(\times\) 10\(^5\) cm\(^{-1}\) mol\(^{-1}\)) and expressed as \(\mu\)mol \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\) protein. The fractional activity of PDHt in PDHn was calculated as (PDHt/PDHt) \times 100. To examine the relation between the fraction of active PDH and its E-1 component, Western blot analysis was conducted using the same mitochondrial preparations.

**Citrate Synthase Assay.** As a standard marker of mitochondrial content in the samples, citrate synthase activity was measured in the mitochondrial pellet. The assay buffer included 50 mM Tris (pH 8.0), 0.5 mM DTNB, 0.1 mM acetyl coenzyme A, 0.05% Triton X-100, and 20 \(\mu\)g mitochondrial protein. The reaction was started by the addition of 0.5 mM oxaloacetate, and absorbance at 412 nm followed for several minutes at 30°C. Citrate synthase activity was determined according to the method of Srere and Matsuoka, which couples coenzyme A to DTNB.

**Measurement of Mitochondrial Respiratory Activity.** The rate of oxygen consumption in freshly isolated liver mitochondria was measured at room temperature using three-channel titration-injection respirometers (Strathkelvin oxygen microelectrodes with Oxygraph [Glasgow, United Kingdom] for data acquisition and analysis). One hundred microliters of the respiratory buffer (250 mM sucrose, 10 mM HEPES, pH 7.2, 20 mM KCl, 5 mM potassium phosphate, and 2 mM MgCl2) was added to each electrode, stirred, and equilibrated for 30 min with air in the oxygraph chambers until a stable baseline was obtained. The mitochondrial suspension was added to give a final concentration of 1 mg/ml protein in 100 \(\mu\)l incubation buffer consisting of respiratory buffer plus substrates/inhibitors. Oxygen flux at the resting state or state 4 respiration through various segments of the electron transport chain (ETC) was determined using (1) flux of electrons from complex I to the end of the ETC (10 \(\mu\)M glutamate and 5 \(\mu\)M malate); (2) flux of electrons from complex II to the end of the ETC (after inhibition of complex I with 5 \(\mu\)M rotenone; state 4 respiration was restored by adding 10 mM succinate as complex II substrate); (3) flux of electrons from complex III to the end of the ETC (inhibition of complex III with 5 \(\mu\)M antimycin A was followed by adding 0.5 mM TMPD and 2 mM ascorbate substrates for complex IV [cytochrome c oxidase, cyclooxygenase]). Oxygen consumption measured in the presence of added ADP (active) was defined as state 3 respiration, and that measured after consumption of ADP (resting) was defined as state 4 respiration. Respiratory control ratio was calculated as ratio of state 3 respiration to state 4 respiration and was used as a marker of mitochondrial respiratory activity. Mitochondrial respiration was calculated as the nanomoles of oxygen per minute per milligram of protein.

**Western Blot Analysis for E-1\(\alpha\) Subunit of PDH, Nitric Oxide Synthase, and Indicators of Apoptosis.** Mitochondria were used for the immunodetection of E-1\(\alpha\) protein, tissue homogenates for nitric oxide synthase and PARP, and cytosol was used for the detection of cytochrome c. The samples for Western blotting were prepared in ice-cold lysing buffer (1:10 [wt/vol]) consisting of phosphate-buffered saline at a pH of 7.4, 230 mg/ml PMSF, 1 \(\mu\)g/ml leupeptin, and 1 \(\mu\)g/ml aprotinin in 1% Triton X-100. After brief sonication and centrifugation (1,000g \times 10 min), these samples were denatured in Laemmli sample buffer. Approximately 15 \(\mu\)g protein was resolved on 10% sodium dodecyl sulfate gel and transferred to the nitrocellulose membrane of 0.45-\(\mu\)m pore size. The membranes were probed with primary mouse monoclonal antibody to either anti E-1\(\alpha\) (dilution, 1:1,000; Molecular Probes, Eugene, OR), anti-nitric oxide synthase (dilution, 1:500; BD Transduction Laboratories, Lexington, KY), anti-cytochrome c (dilution, 1:500; Trevigen, Gaithersburg, MD), or anti PARP (dilution, 1:2,000; Biomol Research Laboratories Inc., Plymouth Meeting, PA). Detection was accomplished using horseradish peroxidase-conjugated antibody and enhanced chemiluminescence reagents (Pierce, Rockford, IL). Ponceau staining of the membranes with transferred proteins showed an equal amount of protein loaded in each lane (data not shown). Negative controls were performed by omitting the primary antibodies. Western blot analysis was performed using Sigma-Stat 3.1 (Aspire Software, Inc., Rockville, MD). Quantitative determinations of signal intensity of the protein bands were performed by measuring the integrated optical density using the Scion image program from Scion Corporation (Fredrick, MD).

**Statistical Analysis** All data are presented as mean \(\pm\) SE or median and range between 25 and 75% distribution. Statistical inference was performed using Sigma-Stat 3.1 (Aspire Software, Inc., Rockville, MD).
Table 1. Hemodynamic and Metabolic Parameters during the Experiments

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time, min</th>
<th>Control (n = 4)</th>
<th>HTS (n = 6)</th>
<th>β-HOB (n = 6)</th>
<th>Pyruvate (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>T0</td>
<td>89 ± 7.4</td>
<td>103 ± 4</td>
<td>98 ± 4</td>
<td>97 ± 5</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>78 ± 3.1</td>
<td>39 ± 2‡</td>
<td>41 ± 3‡</td>
<td>40 ± 2†‡</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>68 ± 5.3</td>
<td>57 ± 3</td>
<td>57 ± 3</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>Blood shed, ml/kg</td>
<td>T0–T60</td>
<td>2.5 ± 0.3</td>
<td>14.5 ± 2.3</td>
<td>10.6 ± 1.4</td>
<td>12.9 ± 1.6</td>
</tr>
<tr>
<td>Osmolality, mEq/l</td>
<td>T0</td>
<td>276 ± 4</td>
<td>283 ± 3</td>
<td>307 ± 2</td>
<td>290 ± 3</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>271 ± 2</td>
<td>278 ± 1</td>
<td>299 ± 3</td>
<td>308 ± 3</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>279 ± 4</td>
<td>305 ± 2</td>
<td>311 ± 2</td>
<td>313 ± 2</td>
</tr>
<tr>
<td>Base excess, mm</td>
<td>T0</td>
<td>4.4 ± 1.6</td>
<td>4.6 ± 1.1</td>
<td>5.5 ± 1.6</td>
<td>5.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>4.1 ± 1.2</td>
<td>6.2 ± 1.2‡</td>
<td>−5.5 ± 1.0‡</td>
<td>−5.1 ± 1.3‡</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>3.8 ± 1.5</td>
<td>−2.5 ± 1.7</td>
<td>−0.6 ± 1.4</td>
<td>11.0 ± 2.5‡</td>
</tr>
<tr>
<td>pH</td>
<td>T0</td>
<td>7.5 ± 0.1</td>
<td>7.3 ± 0.6</td>
<td>7.4 ± 0.3</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>7.3 ± 1.1</td>
<td>7.1 ± 0.4‡</td>
<td>7.1 ± 0.2‡</td>
<td>7.2 ± 0.1‡</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>7.5 ± 0.02</td>
<td>7.2 ± 0.05</td>
<td>7.3 ± 0.01</td>
<td>7.5 ± 0.05*</td>
</tr>
<tr>
<td>Po2, mmHg</td>
<td>T0</td>
<td>89 (88–94)</td>
<td>82 (78–85)</td>
<td>81 (75–84)</td>
<td>78 (73–80)</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>83 (73–91)</td>
<td>103 (97–114)‡</td>
<td>111 (102–128)‡</td>
<td>99 (84–110)‡</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>94 (60–99)</td>
<td>89 (81–91)</td>
<td>81 (74–84)</td>
<td>87 (84–92)</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>T0</td>
<td>40 (35–44)</td>
<td>46 (45–50)</td>
<td>45 (41–48)</td>
<td>47 (45–50)</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>39 (38–42)</td>
<td>32 (29–34)‡</td>
<td>35 (32–37‡)</td>
<td>34 (33–35‡)</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>36 (33–41)</td>
<td>33 (32–38)</td>
<td>41 (33–44)†</td>
<td>51 (46–54)</td>
</tr>
<tr>
<td>Lactate, mm</td>
<td>T0</td>
<td>1.0 (0.8–1.7)</td>
<td>3.1 (2.8–4.5)</td>
<td>1.8 (1.3–2.3)</td>
<td>1.1 (0.9–1.8)‡</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>3.2 (1.1–5.3)</td>
<td>6.0 (5.7–7.4)‡</td>
<td>3.7 (2.8–5.7)‡</td>
<td>6.0 (3.4–6.8)‡</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>4.2 (0.8–7.6)</td>
<td>16.1 (4.0–19.3)</td>
<td>11.1 (5.0–12.2)</td>
<td>8.4 (3.7–15.2)</td>
</tr>
<tr>
<td>Pyruvate, mm</td>
<td>T0</td>
<td>0.11 (0.1–0.2)</td>
<td>0.14 (0.08–0.2)</td>
<td>0.17 (0.08–0.2)</td>
<td>0.13 (0.07–0.13)</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>0.18 (0.1–0.2)</td>
<td>0.24 (0.2–0.3)</td>
<td>0.21 (0.15–0.24)</td>
<td>0.25 (0.19–1.4)</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>0.15 (0.08–0.2)</td>
<td>0.21 (0.2–0.4)</td>
<td>0.28 (0.25–0.32)</td>
<td>1.2 (0.6–1.3)†</td>
</tr>
<tr>
<td>Lactate/pyruvate ratio</td>
<td>T0</td>
<td>9 (8–9)</td>
<td>25 (20–33)</td>
<td>16 (13–18)</td>
<td>12 (9–15)</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>16 (9–24)</td>
<td>28 (26–35)</td>
<td>21 (18–24)</td>
<td>22 (18–25)</td>
</tr>
<tr>
<td></td>
<td>T120</td>
<td>23 (10–26)</td>
<td>51 (19–29)</td>
<td>37 (18–50)</td>
<td>16 (13–22)*</td>
</tr>
</tbody>
</table>

Data are presented as mean SEM except for partial pressure of oxygen (Po2), partial pressure of carbon dioxide (PCO2), and lactate, which are presented as median and interquartile range between 75% and 25% sample distribution. Measurements were done before the start of hemorrhage (T0), at the end of the shock period or control infusion with normal saline (T60), and at the end of the resuscitation or control period (T120). Treatments included no hemorrhage (control), hemorrhage and resuscitation with 7.5% saline (HTS), hemorrhage and resuscitation with β-hydroxybutyrate (β-HOB), and hemorrhage and resuscitation with hypertonic sodium pyruvate (pyruvate).

* P < 0.05, comparing the pyruvate group with HTS or β-HOB for the time-matched data. † P < 0.05, comparing the pyruvate group with controls for the instrumented and time-matched data. ‡ P < 0.05, comparing the before-shock (T0) and after-shock (T60) parameters.

MAP = mean arterial blood pressure.

ware International, Leesburg, VA). Differences between groups for nonrecurring measurements were assessed using one-way analysis of variance. Differences between and within groups for multiple independent samples were performed by Kruskal-Wallis one-way analysis of variance on ranks followed by the Student-Newman-Keuls method. The level of statistical significance was accepted at P < 0.05 after correction for multiple comparisons.

Results

Hemodynamic and Physiologic Response to HS and Resuscitation

Hemodynamic parameters in the control group (group 1) were stable throughout the experiment (table 1). The volume of shed blood for the induction of shock (40 mmHg) did not differ significantly among the various shock groups (groups 2, 3, and 4; table 1; P > 0.05) and was in the range previously reported for the same level (40 mmHg) of HS.19 Because this was a computer-controlled shock model, the volumes of resuscitative fluids infused in each shock group were also similar (table 1). In comparison to baseline values, all animals in the hemorrhaged group had a significant increase in arterial partial pressure of oxygen (Po2). All treatments were equally effective in decreasing Po2 and increasing partial pressure of carbon dioxide (PCO2) (P > 0.05).

Osmolality, Acid–Base Parameters, and Markers of Ischemic Injury

Serum osmolality values were similar in all resuscitated groups. The arterial base excess, a reliable indicator of the severity of HS, oxygen debt, and volume deficit, was negative at the end of the 60-min shock period. Pyruvate administration prevented the magnitude of changes in acid–base parameters that occurred in other treatment groups (P < 0.05). Also, in comparison to controls, hemorrhaged animals showed signs of ischemic conditions characterized by significant lactic acidosis, a low...
lactate/pyruvate ratio, and a decrease in pH ($P < 0.05$) at T60. However, pyruvate treatment was able to reduce these ischemic events when compared with HTS or $\beta$-HOB.

Pyruvate Effects the Release of Hepatic Enzymes and Lipid Peroxidation

The data presented in table 2 indicate that after HS at T120, plasma concentrations of aspartate aminotransferase and alanine aminotransferase were significantly increased in the HTS and $\beta$-HOB groups, and infusion of pyruvate reduced their concentrations, which were similar to those of control operated animals. Also, in these animals, HTS or $\beta$-HOB treatment showed a significant increase in the accumulation of malondialdehyde concentrations in liver tissue when compared with pyruvate or control animals ($P < 0.05$).

Pyruvate Effects on Activation State of Mitochondrial Pyruvate Dehydrogenase and Up-regulation of the E1α Subunit

The data depicted in figure 1 show that in comparison to control operated animals, the activities of both PDH$_{1}$ and PDH$_{2}$ were signiﬁcantly reduced in HTS and $\beta$-HOB groups and not in pyruvate-treated animals ($P > 0.05$). The protective effect of pyruvate on the recovery of PDH activities was also substantiated by the observations that PDH$_{1}$ was $57 \pm 5.9$, $23 \pm 4.5$, $42 \pm 1.8$, and $61 \pm 2.9$% of PDH$_{2}$ in control, HTS, $\beta$-HOB, and pyruvate groups, respectively ($P < 0.05$ between HTS and $\beta$-HOB). The citrate synthase activity in hepatic mitochondria (nmol · min$^{-1}$ · mg$^{-1}$ mitochondrial protein) was approximately similar in all treatment groups (mean ± SE: control, $121 \pm 30.7$; HTS, $129 \pm 25.8$; $\beta$-HOB, $123 \pm 27.5$; pyruvate, $135 \pm 22.3$). The Western blot analysis of 40-kd E1α protein in figure 2 also shows significantly darker protein bands in control and pyruvate groups when compared with HTS- or $\beta$-HOB-treated animals. The quantitative densitometric analysis of this protein also indicates that it was increased in pyruvate-treated animals by factors of approximately 5 and 3 in comparison with the HTS and $\beta$-HOB groups, respectively ($P < 0.05$).
Antimycin A / H11001
Rotenone / H11001
the control group (and a higher energy charge, which was similar to that after pyruvate treatment in comparison with other increase in ADP and decrease in AMP concentrations control animals, liver mitochondria from HTS– and Data presented in table 3 indicate that in comparison to respiratory control ratio with complex specific substrates. variances segments was examined by measuring the respi-

tures reflecting energy metabolites in hepatic tissue after resuscitation are shown in table 2. Data suggests that pyruvate was significantly effective in preventing the loss of hepatocellular ATP in comparison with HTS or β-HOB treatments (percent of controls ATP in HTS, 17%; β-HOB, 33%; pyruvate, 72%, P < 0.05). A concomitant increase in ADP and decrease in AMP concentrations after pyruvate treatment in comparison with other groups resulted in a significantly higher ATP/ADP ratio and a higher energy charge, which was similar to that of the control group (P > 0.05). However, total adenine nucleotide content (ATP plus ADP plus AMP) remained unchanged in all treatment groups.

Pyruvate Effects on Hepatic Adenine Nucleotides Contents and Energy Charge
The changes in adenine nucleotides and purine catabo-
lites reflecting energy metabolites in hepatic tissue after resuscitation are shown in table 2. Data suggests that pyruvate was significantly effective in preventing the loss of hepatocellular ATP in comparison with HTS or β-HOB treatments (percent of controls ATP in HTS, 17%; β-HOB, 33%; pyruvate, 72%, P < 0.05). A concomitant increase in ADP and decrease in AMP concentrations after pyruvate treatment in comparison with other groups resulted in a significantly higher ATP/ADP ratio and a higher energy charge, which was similar to that of the control group (P > 0.05). However, total adenine nucleotide content (ATP plus ADP plus AMP) remained unchanged in all treatment groups.

Pyruvate Effects on Hepatic Mitochondrial Respiration in HS
The capacity of the ETC to oxidize substrates at its various segments was examined by measuring the respira-
tory control ratio with complex specific substrates. Data presented in table 3 indicate that in comparison to control animals, liver mitochondria from HTS– and β-HOB-treated animals showed significantly increased state 4 (resting) respiration. Treatment with pyruvate attenuated these mitochondrial respiratory dysfunctions after HS. Rotenone-sensitive decrease in respiratory control ratio with substrates for complex II and I was significantly low in the HTS and β-HOB groups when compared with pyruvate. In addition, the oxygen consumption was still decreased after complex III inhibition with antimycin (antimycin A plus substrates of complex IV). Our results also demonstrate that respiratory control ratios with complex IV substrates were significantly lower in comparison with the substrates for complex I and II in HTS and β-HOB treatment groups when compared with pyruvate.

Evidence of Decreased Nitric Oxide Synthase, Low Cytochrome c Release, and Decreased Poly-ADP Ribose Polymerase Fragmentation in Liver by Pyruvate in HS
To gain insight into the molecular mechanisms of the action of HTS, β-HOB, and pyruvate on mitochondrial dysfunctions and cell death, inducible nitric oxide synthase, cytosolic cytochrome c, and PARP fragmentation expression were examined in liver from each treatment group. The Western blotting data in figure 3 show significantly lighter protein bands from the pyruvate treat-

Table 3. Effect of Resuscitation Medium on Hepatic Mitochondrial Respiration in Hemorrhagic Shock

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (n = 4)</th>
<th>HTS (n = 4)</th>
<th>β-HOB (n = 5)</th>
<th>Pyruvate (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>State 4</td>
<td>RCR</td>
<td>State 4</td>
<td>RCR</td>
</tr>
<tr>
<td>Glutamate + malate</td>
<td>11 ± 2.3</td>
<td>6.1 ± 1.7</td>
<td>26.7 ± 1.8</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Rotenone + succinate</td>
<td>19 ± 2.9</td>
<td>9.6 ± 3.5</td>
<td>29.8 ± 3.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Antimycin A + ascorbate</td>
<td>14 ± 1.5</td>
<td>3.3 ± 0.7</td>
<td>41.6 ± 4.8</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>+ TMPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM of four to six independent observations. State 4 respiration rates (no added adenosine diphosphate) are reported in nmol O2·min⁻¹·mg⁻¹ mitochondrial protein in the presence of substrates: (1) 10 mM glutamate plus 5 mM malate, (2) 5 mM rotenone plus 10 mM succinate, or (3) 5 mM antimycin A plus 0.5 mM TMPD and 2 mM ascorbate. To induce phosphorylation in state 3, 0.1 mM ADP was added to the reaction mixture containing mitochondria (data not shown). The respiratory control ratio (RCR) was calculated as ratio of state 3 respiration to state 4 respiration and used as a marker of mitochondrial respiratory activity.

* P < 0.05, comparing the pyruvate group with hypertonic saline (HTS) or β-hydroxybutyrate (β-HOB) for the time-matched data.
Table 4. Intensity of Nitric Oxide Synthase, Cytochrome c, and PARP Protein Bands in Response to Various Resuscitative Fluids in Hemorrhagic Shock Rat Liver

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HTS</th>
<th>β-HOB</th>
<th>Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>INOS</td>
<td>0.4 ± 0.07</td>
<td>1.2 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.32 ± 0.02</td>
<td>1.4 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.2 ± 0.01*</td>
</tr>
<tr>
<td>PARP—116 kd</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>PARP—85 kd</td>
<td>0.2 ± 0.08</td>
<td>1.0 ± 0.09</td>
<td>0.9 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>PARP—total</td>
<td>1.7 ± 0.3</td>
<td>2.3 ± 0.1</td>
<td>2.0 ± 0.3</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>% Fragmentation</td>
<td>12 ± 5</td>
<td>43 ± 5</td>
<td>45 ± 6</td>
<td>17 ± 4*</td>
</tr>
</tbody>
</table>

Integrated Optical Density

Data are presented as mean SEM of integrated optical density of immunostained proteins from each treatment group (n = 3). Level of nitric oxide synthase (iNOS) and poly–adenosine diphosphate ribose polymerase (PARP) were examined by Western blotting in tissue homogenates. Cytochrome c was determined in cytosol from the liver samples obtained at 120 min after the start of control or hemorrhage. In comparison with other treatment groups, pyruvate significantly inhibited the expression of iNOS, inhibited the release of cytochrome c from mitochondria into the cytosol, and prevented PARP fragmentation, an apoptotic indicator.

* P < 0.05, comparing the pyruvate group with hypertonic saline (HTS) or β-hydroxybutyrate (β-HOB) for the time-matched data.

Discussion

This study demonstrates the beneficial effects of pyruvate on liver function, hepatocellular injury, mitochondrial functions, and indicators of apoptosis when compared with another ketone body, β-HOB, or saline solutions. In terms of MAP (40 mmHg), amount of blood shed, base excess, pH, PO2, PCO2, and lactate, there were no differences among the various shock groups, indicating that variation in the intensity of HS was not likely the cause of differences in the outcome of various treatments.

Our data strongly suggest a role for pyruvate in preventing liver damage in HS as indicated by a significant decrease in plasma concentrations of liver enzymes aspartate aminotransferase and alanine aminotransferase in comparison to hypertonic saline or β-HOB treatments. This effect of pyruvate was not related to the osmotic effects of the resuscitative fluids because serum osmolality values at the end of the resuscitation period were similar in all animal groups. However, the protective effect of pyruvate in reducing liver injury could be attributed to its antioxidant properties, because free radical scavengers have shown beneficial effects on liver function after ischemia–reperfusion.20 In addition, a significant decrease in lipid peroxidation (accumulation of malondialdehyde in liver) with pyruvate treatment reported in the current study raises the possibility that HS-mediated liver injury is associated with oxidative stress, which can be either a cause or a consequence of mitochondrial dysfunctions.

At low pyruvate concentrations, the availability of pyruvate controls its own decarboxylation, which is not affected by any component of PDH. However, at high pyruvate concentrations, the reaction is strictly controlled by all of the PDH components, especially E-1α.21 The amount of pyruvate we have used in the perfusate is of supramaximal level, and it seems that PDH activity in HS is regulated by the content of E-1α protein. We have observed a threefold higher level of E-1α protein in the pyruvate-treated animals when compared with HTS and β-HOB treatment. These observations show either an increased expression or attenuation in loss of the E-1α protein. The activities of PDH and percentage activation of PDHα reported in the control group are comparatively

Anesthesiology, V 103, No 1, Jul 2005
lower than the values reported for controls in the literature. The underlying mechanisms may be the nutritional state of the animal, techniques of the PDH measurement, and the fact that in our study control group, animals were not totally controlled because they were subjected to the trauma of surgery and anesthesia. Although the results of these experiments cannot account for why these differences occurred, they do contribute toward our understanding of pyruvate utilization via regulation of PDH activity in the liver during HS.

In this study, we have reported a significant decrease of PDH, in HTS- and β-HOB-treated animals when compared with pyruvate. This difference in mitochondrial PDH may indicate oxidative damage of PDH components because citrate synthase (mitochondrial matrix protein), which is more resistant to oxidative stress, was not affected by control or shock treatments. Our in vivo studies have confirmed a positive relation between the decreased mitochondrial PDH and the percentage of studies have confirmed a positive relation between the decreased mitochondrial PDH and the percentage of PARP fragmentation, an indicator of apoptosis, which can be due to increased oxidative stress during HS because pyruvate treatment was able to reverse these changes. In this study, we have provided evidence supporting the notion that increases in PDH by pyruvate can enhance hepatic energy charge. Our results are in accord with those of Jope and Blass, who reported that the proportion of PDH in the active form in both brain and liver mitochondria changed inversely with changes in mitochondrial energy charge. We found that inclusion of pyruvate into the resuscitation fluid can stimulate hepatic metabolism by increasing the pool of high-energy adenine nucleotides, and these pyruvate-mediated changes may have subtle effects on mitochondrial dysfunctions leading to cellular energy depletion. These observations can also explain why pyruvate is an effective protective agent and β-HOB is not, because the entry of β-HOB into the cell is controlled by the availability of cellular ATP, which is depleted in hemorrhagic shock, whereas pyruvate can enhance its own oxidation by stimulating PDH activity.

Availability of mitochondrial substrate, oxygen, and an intact ETC are the key components for adequate ATP production. Our results suggest that in the liver, HS can cause poor flux of electrons from the segments after complex I and II, and also, the defect lies in the transfer of electrons at the terminal end of the ETC (complex IV or cytochrome oxidase). The precise mechanism of respiratory control ratio inhibition at complex IV is not known, but it has been suggested that nitric oxide could inhibit the enzyme cytochrome oxidase by competing with oxygen that can result in the formation of harmful molecules ONOO− (nitrates) and (2) that the loss of cytochrome c is an important substrate for enzyme cytochrome c oxidase (complex IV).

Stimulation of nitric oxide by inducible nitric oxide synthase can also be viewed as a defense mechanism to abate oxidative stress in HS because, in our control study, although nitric oxide synthase was slightly up-regulated, it did not trigger the apoptotic events such as release of cytochrome c and PARP fragmentation. The release of cytochrome c out of the mitrochondrial space into the cytosol is an important trigger for caspase activation, which leads to the events characteristic of apoptosis. Our data show that pyruvate treatment of HS inhibits cytochrome c release from the mitochondria, thereby preventing up-regulation of indicators of cell death such as PARP fragmentation. Rapid activation of this enzyme depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide, thus slowing the rate of glycolysis, electron transport, and subsequently ATP formation. This process can result in cell dysfunction and cell death. Because of the conflicting role of PARP in apoptosis and necrosis, it is difficult to define the HS-mediated cell death by apoptosis or necrosis, but it is clear from our study that inhibition of PARP fragmentation attenuates liver injury and prevents loss of cellular ATP. Thus, pyruvate also mediates its pharmacologic effects, in part by preventing the cleavage of PARP.

In conclusion, the beneficial effects of pyruvate as an alternate substrate in preventing HS-mediated liver injury can be attributed to increases in cellular energetic levels through its antioxidant and antinitrosyl properties and prevention of mitochondrial damage.

The authors thank Mulchand S. Patel, Ph.D. (Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York), for discussion with pyruvate dehydrogenase complex activity, Michael Graham Espey, Ph.D. (Radiation Biology Branch, National Institutes of Health/National Cancer Institute, Bethesda, Maryland), for discussion with inducible nitric oxide synthase study, and Michael J. Dymond, B.S. (Department of Anesthesiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland), for technical help.

References

10. Mongan PD, Capacciione J, West S, Kahaian J, Dubois D, Keneally R, Sharma P: Pyruvate improves redox status and decreases indicators of hepatic

Anesthesiology, V 103, No 1, Jul 2005.
27. Lizzasoain I, Moro MA, Knowles RG, Darley-Usmar V, Moncada S: Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. Biochem J 1996; 314(pt 3):877–80