

HYPOTENSIVE HEMORRHAGE INCREASES CALCIUM UPTAKE CAPACITY AND BCL-X_L CONTENT OF LIVER MITOCHONDRIA

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ABSTRACT—We tested the hypothesis that the response of mitochondrial uptake of calcium and content of Bcl proteins to reversible hemorrhagic shock increases the vulnerability for hepatocellular death. Pentobarbital-anesthetized rats were bled to a mean arterial pressure of 30 to 40 mmHg for 1 h. A subset was then resuscitated (isotonic sodium chloride solution, three times shed volume). Liver mitochondria were isolated at the end of hemorrhage and 1.5 h after the onset of resuscitation. Resuscitation accelerated mitochondrial respiration in the presence of adenosine diphosphate (state 3) above control ($P < 0.01$). The respiratory control ratio ([RCR] state 3/state 4) was calculated using the respiratory rate in the presence of carboxyatractyloside (state 4). The RCR was depressed at the end of hemorrhage and recovered completely in response to resuscitation ($P < 0.05$). The mitochondrial capacity for calcium uptake increased at the end of hemorrhage and remained greater than control ($P < 0.01$) after resuscitation when plasma ornithine carbamoyltransferase (an index of hepatocellular injury) was greater than control ($P < 0.05$). At this time, the capacity for calcium uptake was correlated with plasma ornithine carbamoyltransferase ($r = 0.819$, $P < 0.01$). Mitochondrial content of Bcl-X_L, an antiapoptotic protein, was increased at the end of hemorrhage ($P < 0.03$) with no further change after resuscitation and no change in mitochondrial Bak, a proapoptotic protein. Thus, mitochondrial mechanisms are triggered early during reversible hypovolemia that may limit the intensity of intracellular calcium signaling and its potential to cause cellular injury and death.

KEYWORDS—Bak, hepatocellular injury, fluid resuscitation

INTRODUCTION

Hemorrhagic shock impairs mitochondrial function, and the failure of fluid resuscitation to reverse this impairment is recognized as an important feature of fatal irreversible shock (1, 2). More recently, the mitochondrial accumulation of excess calcium leading to the opening of mitochondrial permeability transition (MPT) pores has been implicated in the initiation of cellular demise (3–5). The mitochondrial uptake of calcium occurs largely through a specialized uniporter in the inner mitochondrial membrane (6). To a point, this uptake can defend against excessive concentrations of cytoplasmic calcium (7). The uptake is driven by the large negative potential within the mitochondrial matrix that is normally maintained by mitochondrial respiration. In the absence of sufficient substrates or during ischemia, the uptake may also be maintained through the hydrolysis of adenosine triphosphate (ATP) (6). However, as the supply of ATP declines, this latter mechanism will fail. Alternatively, when the mitochondrial capacity for calcium uptake is exceeded because of excessive intracellular calcium loads, MPT leads to membrane depolarization and the release of the sequestered calcium, reactive oxygen species, and inhibition of oxidative phosphorylation (3, 4, 7). In the present study, we tested the hypothesis that hemorrhagic shock impairs the capacity of hepatic mitochondria for calcium uptake and alters the mitochondrial balance of Bcl proteins

toward apoptosis. Unexpectedly, we found an increase in uptake capacity during the hypovolemic period that persisted after resuscitation that restored mitochondrial function. This mitochondrial response was proportional to the hepatocellular release of ornithine carbamoyltransferase (OCT) measured after resuscitation, was associated with an increase in mitochondrial Bcl-X_L, and may influence the cellular injury induced by hemorrhage and resuscitation.

MATERIALS AND METHODS

Sprague-Dawley male rats weighing between 300 and 450 grams were housed two to three per cage and kept in a 12-hour light/dark cycle with *ad libitum* access to food and water. Experimental protocols were approved by the University of Maryland Institutional Animal Care and Use Committee. The experiments were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals. For each experiment, two or three rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Femoral venous and arterial catheters were inserted. At least one rat in each study served as a time control by random selection. A rectal temperature probe was placed in this rat to maintain a temperature of 37°C with a servo-controlled heating pad. The remaining rats were placed on the heating pad to experience ambient temperatures similar to the time control. For rats that were hemorrhaged, 20 mL/kg of blood was withdrawn from the femoral arterial catheter in 6 to 9 min. Arterial pressure was monitored thereafter. Additional blood was drawn as needed to maintain mean arterial pressure between 30 and 40 mmHg. At the end of 1 h, rats that were assigned for resuscitation were infused for 15 to 20 min with isotonic sodium chloride solution equal to three times the volume of hemorrhage. The liver was removed through an abdominal incision either after 1 h of hemorrhage or at 1.5 h after the onset of resuscitation. Control rats were not bled and received no resuscitation but experienced the same duration of anesthesia as the hemorrhaged groups. After removal, the liver was immediately placed in homogenization buffer at 4°C.

Isolation of liver mitochondria

Liver was homogenized in 210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 0.5 mg/mL fatty acid-free bovine serum albumin (BSA), and 1 mmol/L ethyleneglycotetraacetic acid (EGTA), pH 7.4 at 4°C based on the method of Holland et al (8). The organ was

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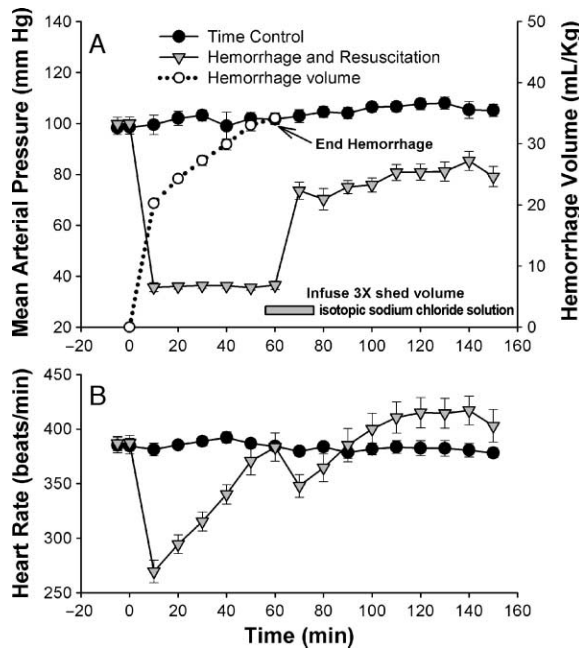


FIG. 1. Mean arterial pressure (A) and heart rate (B) in rats during 1 h of hemorrhage of the volume shown in (A) and resuscitation with isotonic sodium chloride solution and in time controls (hemorrhaged group, $n = 15$; time-control group, $n = 17$).

rinsed three to four times with this buffer before it was minced with scissors. The pieces were homogenized with four strokes in a motorized Potter-Elvehjem homogenizer and centrifuged (Eberbach Corp., Ann Arbor, MI) in a Sorvall SS34 rotor (Thermo Electron Corp., Asheville, NC) for 3 min at 1,698g. Excess fat was removed from the supernatant that was then centrifuged for 12 min at 8,884g. The supernatant was discarded, and a small amount of buffer was added to each tube. The pellets were resuspended, and the tubes were topped off with buffer before being centrifuged for 12 min at 14,763g. The pellets were resuspended and washed in the same buffer but without EGTA. The washed mitochondria were concentrated by centrifugation for 12 min at 14,763g. They were then resuspended in buffer without EGTA and kept at 4°C.

Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured with a Clark-type oxygen electrode (Hansatech, Norfolk, UK) at 30°C. The protein content of the mitochondrial suspensions was measured using the biuret reagent. Mitochondria were suspended at 1 mg of protein/mL in 125 mmol/L KCl, 2 mmol/L K_2HPO_4 , 20 mmol/L HEPES (pH 7.0), 1 mmol/L MgCl, 0.4 mg/mL BSA, and 0.25 mmol/L EGTA, with 10 mmol/L succinate as substrate or the combination of 5 mmol/L glutamate and 5 mmol/L malate. To determine the state 3 rate of respiration, 0.1 or 0.2 mmol/L potassium-adenosine diphosphate (ADP) was added. Measurement continued until the rate of respiration declined with the depletion of ADP. Then 2 μ mol/L carboxyatractylsido was added to block the exchange of any ADP formed external to the mitochondrial matrix with newly formed ATP, preventing ATP synthesis and causing the minimal state 4 rate of respiration.

Measurement of Ca^{2+} uptake capacity of mitochondria

Mitochondrial uptake of Ca^{2+} was measured in a Spex 1681 0.22 m spectrofluorometer (Horiba Jobin Yvon, Inc., Edison, NJ) at 37°C with stirring using excitation and emission wavelengths of 506 and 531 nm, respectively (9). Measurements were made in 2.5 mL of buffer containing 125 mmol/L KCl, 2 mmol/L K_2HPO_4 , 20 mmol/L HEPES (pH 7.0), 1 mmol/L MgCl, 0.4 mg/mL BSA, 5 μ mol/L EGTA, and 5 μ mol/L calcium green dye with several substrates and 3 mmol/L Mg^{2+} -ATP. Repeated additions of 200 nmol of $CaCl_2$ in 5 μ L were made at 3, 6, 9, and 12 min after the mitochondria were introduced into the measuring cuvette at a concentration of 0.25 mg of protein/mL.

Measurement of OCT

Plasma OCT was measured in arterial samples collected with heparin as an anticoagulant. Samples were stored at -70°C and then assayed by the method of Ohshita et al. (10), modified to use less volume (20 μ L per sample). Enzymatic activity was determined from the ability of each sample to catalyze the production of citrulline from substrate with 1 unit defined as 1 μ mol of citrulline per min at

37°C. The OCT occurs only in trace amounts in nonhepatic tissue, and its plasma concentration parallels the standard enzymatic markers for hepatocellular injury in this experimental model of hemorrhage and resuscitation (11, 12).

Measurement of mitochondrial proteins

Mitochondrial suspensions containing 500 μ g of total protein were lysed on ice with 125 μ L of radio immunoprecipitation assay buffer (Upstate Cell Signaling Solutions, Lake Placid, NY) with protease inhibitors. Particulates were removed by centrifugation for 10 min at 12,000g. The protein content of the supernatants was determined by the biuret method, and 10 μ g of total protein per sample was separated on sodium dodecyl sulphate-polyacrylamide gels by electrophoresis. The separated samples were transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad, Hercules, Calif). Membranes were blocked overnight with 5% nonfat milk at 4°C, rinsed and incubated for 1 h at room temperature in polyclonal antibodies from rabbits to Bcl- x_L (1:20,000; Pharmingen, San Diego, Calif; Cat no. 610211) or Bak (1:5,000; Upstate Cell Signaling Solutions; Cat no. 06-536). The blots were then rinsed and incubated for 1 h at room temperature in peroxidase-conjugated antibody to rabbit IgG from goat (1:10,000; Vector Laboratories, Burlingame, Calif; Cat no. PI-1,000). Immunoreactive bands were detected using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ; Cat no. RPN2106). The antibodies were then removed from the blots with stripping solution (Pierce, Rockford, Ill; Cat no. 21059). Immunological staining was repeated as described above but using a primary monoclonal antibody to voltage-dependent anion channel (Vdac). (Anti-Porin 31 HL 1:40,000; EMD Biosciences, San Diego, Calif; Cat no. 529534) followed by peroxidase-conjugated antibody to mouse IgG from horse (1:10,000; Vector Laboratories; Cat no. PI-2000). Immunoreactive bands were scanned, and integrated optical densities were measured with a Bioquant (Nashville, Tenn) image analysis system. Results for

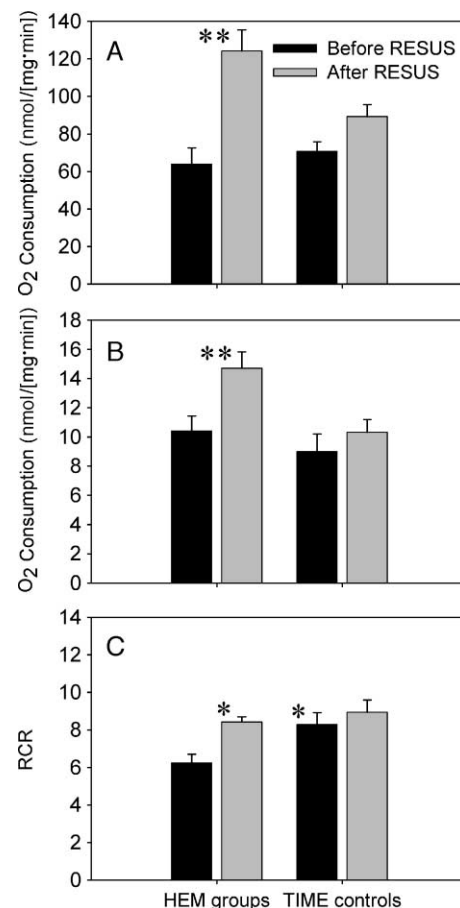


FIG. 2. Resuscitation after hemorrhage accelerates the rate of respiration of liver mitochondria oxidizing 10 mmol/L succinate after addition of ADP to cause state 3 (A) and after addition of 1 μ mol/L carboxyatractylsido to elicit state 4 (B). C, The RCR (state 3/state 4) also increases with resuscitation. Time-control groups were neither bled nor resuscitated but experienced the same duration of anesthesia and surgery as HEM groups. **Difference from all other values, $P < 0.01$. *Difference from before RESUS in the HEM group, $P < 0.05$. Error bars are SEM. $n = 7$ to 10 per group. HEM indicates hemorrhage; RESUS, resuscitation.

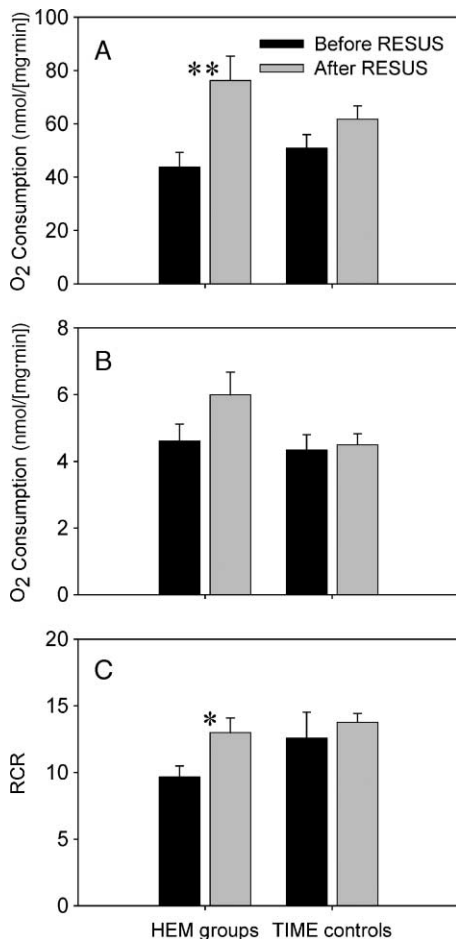


FIG. 3. Resuscitation after hemorrhage accelerates state 3 respiration measured after addition of ADP (A) of liver mitochondria oxidizing 5 mmol/L glutamate and 5 mmol/L malate with no change in state 4 measured after addition of 1 μ mol/L carboxyatractyloside (B). C, The RCR (state 3/state 4) also increases with resuscitation. Time-control groups were neither bled nor resuscitated but experienced the same duration of anesthesia and surgery as HEM groups. **Difference from all other values, $P < 0.05$. *Difference from before RESUS in the HEM group, $P < 0.05$. Error bars are SEM. $n = 7$ to 10 per group. HEM indicates hemorrhage; RESUS, resuscitation.

Bcl-x_L and Bak were normalized to V_{dac} to correct for differences in mitochondrial protein loading. Results were tested statistically by analysis of variance (ANOVA) (13) and for the presence of correlations among variables (14).

RESULTS

Hemodynamic response to hemorrhage and resuscitation

Hemorrhage led to an initial bradycardia that subsided steadily throughout the first hour of hypotension (Fig. 1B) as blood was withdrawn progressively to maintain the mean arterial pressure below 40 mmHg (Fig. 1A). Resuscitation led to significant partial recovery of arterial pressure. During the infusion of isotonic sodium chloride solution, heart rate declined from its value at the end of hemorrhage but rebounded after the infusion ended to values above control during the 40 min before the liver mitochondria were harvested. Figure 1 shows results for rats that were resuscitated after 1 h of hemorrhagic hypotension and their matched control group. Similar responses occurred in the groups in which the mitochondria were harvested after 1 h of hemorrhage with no resuscitation and are not shown. The average

total volume removed in all of the experiments to maintain the mean arterial pressure between 30 and 40 mmHg was 33.2 ± 0.6 mL/kg.

Oxygen consumption in liver mitochondria after hemorrhage and resuscitation

At the end of hemorrhage before resuscitation, the rates of respiration for all substrates tested did not differ significantly from the rates measured in time controls (Figs. 2, A and B, and 3, A and B). However, the respiratory control ratio ([RCR] state 3/state 4) with succinate as the substrate was less than its respective time control ($P < 0.05$, Fig. 2C). A similar trend was observed with glutamate and malate as substrates but was not significant (Fig. 3C). Resuscitation in the hemorrhaged group then led to significant increases in both state 3 and state 4 consumption of oxygen when succinate was the substrate that exceeded the respective time-control values (Fig. 2, A and B). With glutamate and malate as substrates, resuscitation in the hemorrhaged groups led to a significant increase in the state 3 consumption of oxygen, with no change in state 4 (Fig. 3, A and B). Oxygen consumption did not change significantly in the time-control groups for any

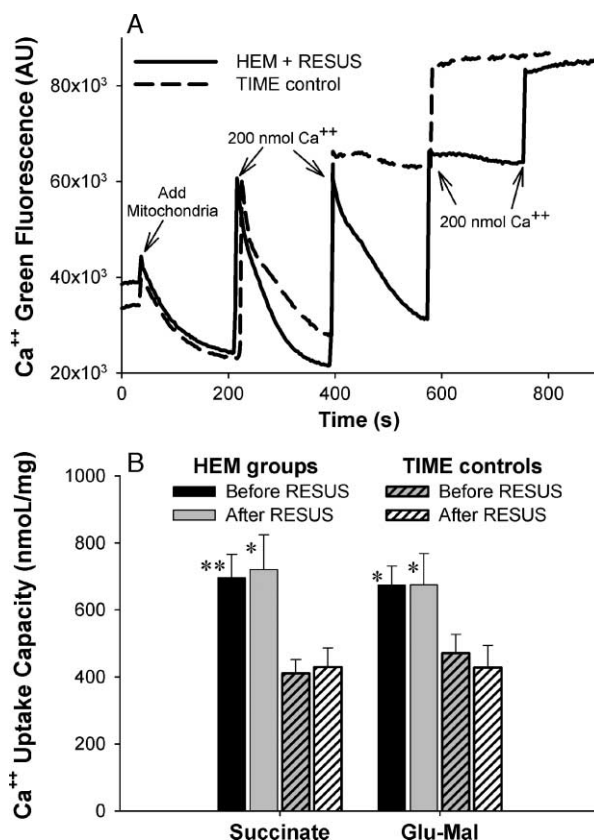


FIG. 4. A, Representative examples of mitochondrial uptake of calcium as assessed by the fluorescence of calcium green dye. Fluorescence increases with the initial addition of mitochondria and with subsequent additions of 200 nmol of Ca²⁺ and then decreases as Ca²⁺ is sequestered by the mitochondria. B, With either 10 mmol/L succinate or the combination of 5 mmol/L glutamate and 5 mmol/L malate as substrates, the total capacity for mitochondrial Ca²⁺ uptake is greater in the hemorrhaged groups both before and after resuscitation than in time controls, * $P < 0.05$ and ** $P < 0.01$. Time-control groups were neither bled nor resuscitated but experienced the same duration of anesthesia and surgery as HEM groups. Error bars are SEM. $n = 6$ to 7 per group. HEM indicates hemorrhage; RESUS, resuscitation.

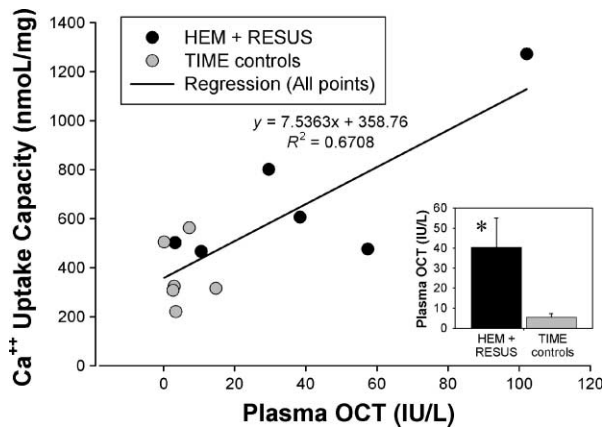


FIG. 5. Relationship of calcium uptake capacity of liver mitochondria oxidizing 10 mmol/L succinate in the presence of rotenone to plasma OCT after hemorrhage and resuscitation and in matched time controls. The slope of the regression line and correlation is significant, $P < 0.05$. *Inset shows plasma OCT is increased by HEM and RESUS, $P < 0.05$. HEM indicates hemorrhage; RESUS, resuscitation.

substrate tested (Figs. 2, A and B, and 3, A and B). The changes in respiration in the hemorrhaged groups that followed resuscitation were accompanied by increases in the RCR for all substrates tested (Figs. 2C and 3C, $P < 0.05$).

Response of mitochondrial capacity for Ca^{2+} uptake and its relation to plasma OCT

Representative traces showing the uptake of calcium by liver mitochondria show greater uptake in a sample taken after hemorrhage and resuscitation than after a matched time control (Fig. 4A). Note that very little uptake occurred after the second addition of 200 nmol of Ca^{2+} to control mitochondria compared with the mitochondria from the hemorrhaged rat. Both before and after resuscitation, the mitochondria from the hemorrhaged groups showed greater capacity for the uptake of calcium than did time controls when either succinate or the combination of glutamate and malate were used as substrates (Fig. 4B). Similar results were obtained when succinate was used in the presence of rotenone. In rats that were resuscitated after hemorrhage, plasma OCT was greater than in the time-matched control rats ($P < 0.05$, Fig. 5). Furthermore, the mitochondrial uptake of calcium in all of these rats showed a significant correlation with the plasma OCT for all substrates tested ($r = 0.798$ for succinate,



FIG. 6. Representative immunoblots show mitochondrial Bcl- x_L and Bak after hemorrhage before and after resuscitation and in matched time controls. Mitochondrial Vdac is also shown to indicate total loading of mitochondrial protein. HEM indicates hemorrhage; RESUS, resuscitation.

0.819 for succinate with rotenone, and 0.777 for glutamate and malate) as shown in Figure 5 for succinate in the presence of rotenone.

Mitochondrial content of Bcl- x_L increased after hemorrhage

A second set of experiments following the same protocol was used to determine the effect of hemorrhage and resuscitation on the mitochondrial content of selected proteins known to regulate cell death pathways. The antiapoptotic protein, Bcl- x_L , detected on Western blots was greater than that of control after hemorrhage ($P < 0.03$) and did not change with resuscitation (Figs. 6A and 7A). The proapoptotic mitochondrial protein, Bak, did not differ from that of control after either hemorrhage or resuscitation (Figs. 6B and 7B). As a result, the ratio of mitochondrial Bak to Bcl- x_L was reduced compared with that of control at the end of hemorrhage, and this decrease persisted after resuscitation (Fig. 7C, overall treatment effect by ANOVA, $P < 0.05$). In a subset of mitochondrial samples that were available from the experiments done to measure calcium uptake capacity at the end of hemorrhage before resuscitation, we determined the Bcl- x_L content and found it to be greater

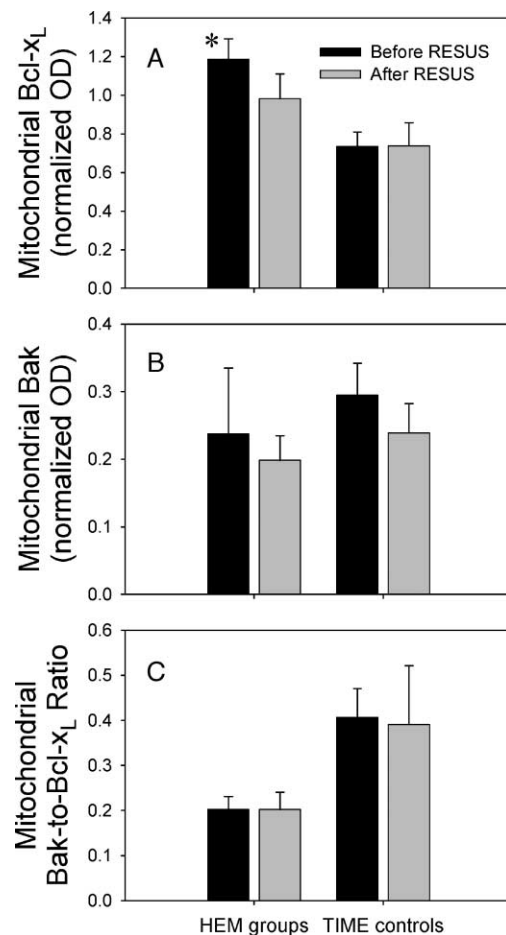


FIG. 7. Mitochondrial Bcl- x_L (A), Bak (B), and their ratio (C) in response to hemorrhage and resuscitation. The OD of Bcl- x_L and Bak bands were normalized to the OD of Vdac. Time-control groups were neither bled nor resuscitated but experienced the same duration of anesthesia and surgery as HEM groups. *Difference from time control, $P < 0.03$. C, The HEM groups differ from controls by ANOVA, $P < 0.05$. Group A, $n = 8$; group B, $n = 6$; group C, $n = 6$. HEM indicates hemorrhage; OD, optical density; RESUS, resuscitation.

than that of control after hemorrhage ($P < 0.04$). However, the measured Bcl-x_L in this experimental subset was not correlated with the measured capacity for calcium uptake for any of the mitochondrial substrates tested ($r = 0.127 - 0.331$; succinate, $n = 14$; succinate with rotenone, $n = 12$; glutamate and malate, $n = 12$). We also prepared immunoblots for Bcl-x_L from whole liver homogenates, but the presence of multiple bands in the expected region precluded a definitive determination of whether the total liver content of this protein was upregulated by hemorrhage.

DISCUSSION

Mitochondrial dysfunction leading to inadequate supplies of high-energy phosphates to support cellular metabolism has long been regarded a central event in the etiology of hemorrhagic shock. However, only severe and prolonged hypovolemia causes sufficient mitochondrial defects to prevent their reversal in response to resuscitation (1, 2). Accordingly, other mechanisms such as inflammatory processes that influence cellular and organ function during the recovery from hemorrhagic shock have received recent attention. Nonetheless, mitochondria also participate in the handling of intracellular calcium and in the control of cellular viability through their interaction with the Bcl-2 family of proteins. The role of these processes in the cellular responses to hemorrhage and resuscitation has not been fully elucidated.

In the present study, resuscitation with isotonic sodium chloride solution using three times the volume of hemorrhage led to partial recovery of arterial pressure and prevented lethality for the duration of the experiment. Liver mitochondria showed a modest functional deficit at the end of 1 h of hemorrhage, as reflected by a small decrease in RCR that indicates reduced efficiency of oxidative phosphorylation because of the leakage of hydrogen ions into the mitochondrial matrix. This reduction occurred before the release of OCT that was shown not to occur until after resuscitation in a previous study (15). The modest response of RCR appears consistent with follow-up measurements of nitrotyrosine and protein carbonyls in leftover mitochondrial suspensions, suggesting no significant oxidative damage as a result of either hemorrhage or resuscitation (data not shown).

The RCR showed complete recovery at 1.5 h after resuscitation. However, the increase in plasma OCT suggested modest hepatocellular injury that others have documented to include scattered foci of edema and occasional areas of focal vascular occlusion at 2 h after resuscitation of a hemorrhage of similar magnitude but with a longer duration and significant reuptake of shed volume (11). After resuscitation in this latter model, nitrotyrosine is expressed in hepatic cells at 4 h (16), and survival at 24 h is less than 50%. Whether hepatocellular changes reverse the early recovery of mitochondrial function in our model remains to be determined. However, the rats in our study showed no reuptake of shed blood before the scheduled resuscitation at 1 h, suggesting a better rate of long-term survival.

The early recovery of RCR after resuscitation was accompanied by an accelerated rate of state 3 respiration that could involve action of second messengers on the electron

transport chain, such as that reported for cyclic adenosine monophosphate (cAMP) on complex 1 (17) or other ATP-dependent mechanisms such as we have recently reported in mitochondria from the lung (18). In a similar study of pressure-controlled hemorrhage, mitochondrial redox state was restored to values above control when resuscitation with shed blood was begun before the reuptake phase (2).

Despite the modest response of RCR, we tested the hypothesis that hemorrhagic shock limited the ability of liver mitochondria to sequester calcium. We found, on the contrary, that the capacity for calcium uptake increased significantly during hemorrhage and was maintained after resuscitation. This response may enhance the mitochondrial buffering of cytoplasmic calcium during hemorrhage and thus reduce the vulnerability of the mitochondria to MPT that could initiate cell death (7).

It was possible that a subpopulation of mitochondria was preferentially damaged by hemorrhage, so that it was not isolated in the experimental groups, causing the net uptake of the remaining mitochondria to exceed that in the time-control groups. Assuming zero capacity for calcium uptake in this hypothetical subpopulation, we calculated that the net recovery of mitochondria after hemorrhage would need to be 40% less than in controls to account for our findings. After suspending the final mitochondrial pellets in matched equal volumes of buffer, there was no difference in protein concentration with the hemorrhage values averaging $98\% \pm 6\%$ of control. Furthermore, equal amounts of protein loaded onto immunoblots for Vdac led to similar staining (Fig. 6). Thus, we have no evidence that the mitochondrial recovery or purity was different between the control and hemorrhaged groups.

The elevation in calcium uptake capacity observed at the end of hemorrhage was similar to that reported in brain mitochondria by Rosenthal et al. (19) after 100 min of reperfusion that followed cerebral ischemia caused by 10 min of cardiac arrest. Thus, the brief ischemia in their study triggered mechanisms that led to an increase in mitochondrial uptake capacity during the subsequent period of spontaneous circulation. To the extent that similar mechanisms were present during the hour of hypovolemia in our study, our findings suggest that the hepatic perfusion was adequate to support such a response. Treatment of cells with dibutyryl cAMP reduces the leakage of mitochondrial Ca²⁺ (20), and direct incubation of isolated kidney mitochondria with dibutyryl cAMP increases Ca²⁺ uptake and retention (21). Similar second-messenger effects may be triggered by the hormonal response to hemorrhage that includes increases in circulating epinephrine and glucagon (22). Such effects are consistent with the observed correlation between the calcium uptake capacity and plasma OCT. The responses of OCT and hormones to hemorrhage (22) increase with the severity of circulatory shock. Enhanced mitochondrial capacity for Ca²⁺ uptake may then buffer the increases in cytoplasmic Ca²⁺ mediated by hormones, such as vasopressin (23) and glucagon (24), in response to hemorrhage (22, 25). Additional protection of mitochondria could be provided by hemorrhage-evoked release of glucocorticoids (22) through the induction of heat shock protein 60 (26).

Antiapoptotic proteins of the Bcl-2 family might also account for the increased capacity for calcium uptake of liver mitochondria. A neuronal cell line that overexpresses Bcl-2 was shown by Murphy et al. (7) to have greater mitochondrial capacity for calcium uptake and state 3 consumption of oxygen than its parent line. Although mitochondrial Bcl-2 was not detected in preliminary trials from our experiments, the related peptide Bcl-x_L increased in the mitochondria at the end of hemorrhage as did the mitochondrial capacity for calcium uptake. However, accelerated rates of state 3 oxygen consumption and improved RCR were not observed until after fluid resuscitation. Thus, the early increase in calcium uptake capacity at the end of hemorrhage did not parallel the respiratory response and did not result from an increase in oxidative phosphorylation, but could still depend on the hydrolysis of the cytosolic concentration of ATP included in the buffer. Dissociated responses of respiration and calcium uptake in the presence of ATP have also been observed in brain mitochondria. After 10 min of cerebral ischemia, there is a marked reduction in the state 3 consumption of oxygen, yet the capacity for calcium uptake is maintained (19). In the present study, the changes in Bcl-x_L at the end of hemorrhage showed no correlation with the calcium uptake capacity, suggesting that these acute mitochondrial responses occurred independently.

We were not able to determine the source of mitochondrial Bcl-x_L during the hemorrhage. Saitoh et al. (27) found that during the first hour of reoxygenation after prolonged anoxia, Bcl-2 protein increases in fibroblasts transfected with the Bcl-2 gene. The time course and magnitude of this increase is similar to the change in Bcl-x_L reported here. Hemorrhage of the magnitude used in the present study reduces the hepatic energy charge by less than 50% (28) but may not eliminate *de novo* synthesis of Bcl-x_L. The increase in mitochondrial Bcl-x_L could also reflect a change in its intracellular trafficking. The FK506 binding protein, FKBP38, is reported to translocate and anchor Bcl-x_L to the mitochondrial membrane (29). The upregulation of FKBP38 is antiapoptotic (29), and this characteristic of FKBP38 may be modulated by interactions with Ca²⁺ and calmodulin (30). Finally, sympathectomized mice fail to maintain hepatic concentrations of Bcl-x_L during challenge with a Fas agonist and show increased hepatic apoptosis and mortality (31). Thus, the sympathetic response to hemorrhage (22) may play an important role in the rapid increase in mitochondrial Bcl-x_L that we observed.

As with Bcl-2, mitochondrial Bax was not detected consistently in preliminary trials. The constitutive proapoptotic protein Bak was present in mitochondria but did not change in response to hemorrhage or resuscitation. The increase in the mitochondrial content of the antiapoptotic protein Bcl-x_L in proportion to Bak after hemorrhage may reduce the likelihood of mitochondrially mediated apoptosis and, thus, modulate the normal turnover of hepatic cells by preserving a subpopulation that is nearing the end of its programmed life span to compensate for cells that succumb to necrosis because of perfusion-related hypoxia or to lethal intracellular calcium loads. The potential benefit of an increase in mitochondrial Bcl-x_L is supported further by the report of Mongan et al. (32) that resuscitating pigs in

hypovolemic shock with hypertonic sodium pyruvate augments the hepatic expression of Bcl-x_L, improves hepatic redox state, reduces hepatocellular apoptosis, and delays cardiovascular decompensation.

Our results are then consistent with the hypothesis that the early response to hemorrhagic hypotension includes mitochondrial changes that help to preserve the function of these organelles in the face of reduced oxygen tension and substrate supply. The responses may be similar to compensatory responses of liver mitochondria to endotoxin-induced shock in rats where increases in state 3 consumption of oxygen (33) and other vital mitochondrial proteins (34) have been observed. The changes that we have described can defend, at least transiently, against hepatocellular injury caused by calcium overload and apoptosis. However, it is increasingly clear that the mechanisms that control cellular viability are complex. Thus, the observed responses may also increase in hepatocellular vulnerability to other modes of destruction such as autophagic cell death, as discussed in a recent review (35). Accordingly, the full implications of these observations will require additional investigation.

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