

Activation of poly(ADP-ribose) polymerase in severe hemorrhagic shock and resuscitation

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Watts, John A., Robert M. Grattan II, Bradley S. Whitlow, and Jeffrey A. Kline. Activation of poly(ADP-ribose) polymerase in severe hemorrhagic shock and resuscitation. *Am J Physiol Gastrointest Liver Physiol* 281: G498–G506, 2001.—This study examines activation of poly(ADP-ribose) polymerase (PARP) in the ileum during hemorrhage and resuscitation and determines if inhibition of PARP reduces organ dysfunction and metabolic acidosis. Awake, nonheparinized rats were hemorrhaged (40 mmHg, 60 min). Resuscitation used Ringer's solution ($2\frac{1}{3} \times$ shed volume) and packed red blood cells ($\frac{2}{3}$ shed volume). Ileal PARP activity was elevated at the end of hemorrhage (3.6-fold) and 10 min of resuscitation (5-fold). The subsequent decline in PARP activity observed after 60 min of resuscitation was not due to cleavage by caspase-3. Ileum permeability increased 10-fold and circulating liver enzymes increased 4- to 6-fold following 60 min of resuscitation in animals pretreated with 3-aminobenzoic acid, a structural analog that does not inhibit PARP. Pretreatment with 3-aminobenzamide (3-AB), a PARP inhibitor, reduced these changes, whereas posttreatment with a bolus of 3-AB was ineffective. Metabolic acidosis, accumulation of lactate, and base deficit was reduced by pretreatment with 3-AB. PARP is activated in the ileum by hemorrhage and by resuscitation. Activation of PARP contributes to organ dysfunction in the ileum and liver and appears to be central to the development of metabolic acidosis.

multiorgan failure; ileum; gut permeability; 3-aminobenzamide; caspase

WHEN ACTIVATED BY DNA single strand breaks, poly(ADP-ribose) polymerase (PARP) cleaves NAD^+ and attaches polymers of ADP-ribose to proteins. PARP normally participates in homeostatic processes (12). In stress conditions, however, excess activation may lead to depletion of NAD^+ , inhibition of glycolysis, depletion of ATP, and necrotic cell death (2–4, 7, 16, 27–29, 32, 50).

Previous studies of hemorrhagic shock have implicated PARP as a mediator of organ injury in hemorrhage and resuscitation by showing beneficial effects of pharmacological inhibition or by employing mice that are genetically deficient in PARP. Beneficial effects of PARP inhibition in models of hemorrhage and resuscitation include improved mean arterial pressure (MAP) following resuscitation in an underresuscitated model (31, 33), improved vascular contractile responses (30),

and reduced organ injury in liver, kidney, and pancreas (21, 22). Knockout mice that lack PARP activity also show a less severe decline in MAP following hemorrhage and resuscitation (19). None of these studies has directly measured the time course of PARP activation, the role of PARP activation in development of increased ileum permeability, or the metabolic effects of PARP activation during the hemorrhage period. Therefore, the purposes of the present study were threefold: first, to characterize the general time course of PARP activation during hemorrhage and resuscitation in the ileum by using a direct enzyme assay technique; second, to determine if inhibition of PARP activity influences the recovery of organ function; and third, to assess the effects of PARP activation on the development of metabolic acidosis during hemorrhage and subsequent resuscitation.

MATERIALS AND METHODS

These experiments were performed in adherence with the National Institutes of Health guidelines on the use of experimental animals. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Carolinas Medical Center.

Instrumentation

The studies were performed with male Sprague-Dawley rats (300–450 g) that had ad libitum access to Teklad rat chow and water before the experiments. The animals were anesthetized with isoflurane (induction with 3–4% and maintenance with 1–2% isoflurane, balance oxygen) with a small animal anesthesia machine and ventilator. The femoral triangle was prepared with aseptic conditions, and the femoral artery was cannulated with a short segment of PE-50 tubing attached to PE-90 tubing to facilitate blood withdrawal and to monitor blood pressure. A PE-50 catheter was inserted into the femoral vein for resuscitation. Catheters were filled with 101% of their previously determined dead space volume with a sterile solution of 1 U heparin/ml of saline and were routed and exteriorized at the back. After the incisions were sutured, the animals were awakened and regained consciousness for 30 min in a clear chamber made of Plexiglas. The arterial catheter was connected to monitor hemodynamics with a Statham transducer (Statham/Gould Instruments, Millersville, MD) and for digital data acquisi-

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tion (Acknowledge software, Biopac Systems, Santa Barbara, CA). Values of MAP are presented from the baseline period, the end of the hemorrhage period, and at the end of each phase of the resuscitation period.

Hemorrhage and Resuscitation

Hemorrhage was initiated in conscious rats by withdrawing blood from the femoral artery into a syringe containing anticoagulant citrate dextrose (10% vol/vol) at a rate of 1 ml/min with a syringe withdrawal pump (Harvard Apparatus, Holliston, MA). Animals were bled to a MAP of 40 mmHg. Additional blood was withdrawn, or aliquots of Ringer's solution were added as required to maintain animals at 40 mmHg for 60 min. Animals were then resuscitated with 1× the shed blood volume as Ringer solution at a rate of 1 ml/min and 2/3 of the shed volume as packed red blood cells (PRBC) at a slower rate (0.5 ml/min) to minimize potential changes in blood ions (5). Rats then received 1 1/3 times the shed volume as Ringer solution (1 ml/min) followed by additional Ringer solution (5 ml·kg⁻¹·h⁻¹) until 60 min after resuscitation was initiated. The use of PRBC was designed to mimic the procedure most often used for resuscitation of severe hemorrhagic shock in emergency departments and trauma surgery units.

The blood that was shed during hemorrhage was weighed and centrifuged at 1,500 *g* for 10 min. The plasma was removed by aspiration, and the PRBC were resuspended in Ringer solution just before reinfusion. The PRBC solution was gently agitated and temperature controlled until 2/3 of the shed volume of resuspended PRBC was reinfused during resuscitation.

PARP Assays

Animals were anesthetized with pentobarbital (15 mg/kg iv), and tissues were removed for the measurement of PARP activity after the sham protocol, following hemorrhage with no resuscitation, following 10 min of resuscitation with the initial Ringer solution, and 60 min after the initiation of resuscitation. Tissues were freeze-clamped in tongs cooled in liquid nitrogen and were stored at -70°C until assay. These samples were homogenized (Polytron homogenizer, 10-s burst at medium setting) in ice-cold buffer containing 25 mM MgCl₂, 50 mM Tris, pH 8.0, 10 μM β-mercaptoethanol, 20 μl protease inhibitor cocktail P8320 (Sigma), 0.5 μM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 3,000 *g* for 5 min. The supernatant was removed, and the nuclei were resuspended in 0.5 ml of homogenizing buffer as described above with the addition of Triton X-100 (0.01%) to lyse the nuclei. PARP reactions were performed with a commercially available PARP activity assay kit (TA5334; R & D systems, Minneapolis, MN). The reactions contained PARP buffer (10 μl), NAD⁺ (1 mM, 10 μl), ³²P-NAD⁺ (2–4 μl, 30 Ci/mmol, 2 μCi/μmol; New England Nuclear Life Science Products, Boston, MA), and distilled H₂O to make 80 μl total volume. Reactions were initiated with the addition of 20 μl of the nuclear extract. The PARP inhibitor 3-aminobenzamide (3-AB; 6 μl of 200 mM stock solution, final concentration 12 mM) was added to the reaction mixture before initiating the reaction with the nuclear extracts to inhibit PARP activity. Reactions were terminated after 5 min by the addition of ice-cold 20% TCA (900 μl). Reactions were vacuum filtered with 10-cm GF/C filters that were prerinsed with 10% TCA. Filters were washed consecutively with 80 ml of ice cold 10% TCA and 40 ml of 95% ethyl alcohol. Radioactivity trapped on the filters was determined with a scintillation counter (Beckman model

LS6000IC). Blank reactions were run with no nuclear extract, and this activity was subtracted from all reactions. Specific activity of the NAD⁺ solution was determined by placing 1 μl of the blank reaction onto a GF/C filter and counting its radioactivity. Protein content of the nuclear extracts was determined with a micro-Lowery assay kit (Sigma). Activity was expressed as picomoles per minute per milligram protein.

Western Blotting of PARP

Nuclei isolated from the ileum samples as in the PARP enzyme assay or human cells in culture were suspended in lysis buffer [10% (vol/vol) glycerol and 2% (wt/vol) SDS in 83 mM Tris, pH 6.8] and sheared by four passages through a pipette. To part of the homogenate 10% β-mercaptoethanol and 0.05% bromophenol blue were added, and lysates were boiled for 5 min and stored at -80°C until immunoblotting was performed. Protein was determined by using a micro-Lowery assay kit. Equal amounts of protein were resolved on 12% SDS-polyacrylamide gel and electrotransferred to 0.45-μm Hybond ECL nitrocellulose membranes (Amersham Life Sciences, Little Chalfont, UK) by the wet transblot method in transfer buffer [0.025 M Tris, 0.192 M glycine, 2.6 mM SDS, and 20% (vol/vol) methanol, pH 8.3] at 100 V for 1 h. Blots were blocked overnight at 4°C with blocking buffer (PBS with 0.1% Tween) containing 5% fat-free milk powder (Carnation, Glendale, CA). After being rinsed 3 times for 5 min each in PBS containing 0.1% Tween 20, blots were incubated for 1 h at room temperature with 2.0 μg/ml of primary antibody (PARP N-20; Santa Cruz Biotechnology, Santa Cruz, CA). After being rinsed as above, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody diluted 1:1,000 in blocking buffer with constant agitation. Immunoblots were rinsed again as above and detected via an enhanced chemiluminescence method (ECL Western Blotting detection system; Amersham Life Science). Autoradiographic film (X-OMAT AR; Eastman Kodak, Rochester, NY) was exposed to immunoblots for 2, 5, and 10 min to obtain satisfactory images.

Caspase-3 Assay

Animals were anesthetized with pentobarbital (15 mg/kg iv), ileum samples were removed, and tissues were freeze-clamped in tongs cooled in liquid nitrogen and stored at -70°C until assay. Caspase-3 activity was then measured by modification of a previously published method (17). Tissue samples were homogenized (Polytron homogenizer) in isolation buffer (10 mM MgCl₂, 0.25% Nonidet P-40 Igepal) and centrifuged (average 115,000 *g* × 30 min). Supernatants were mixed 1:1 with storage buffer (40 mM HEPES, pH 7.1, 20 mM NaCl, 2 mM EDTA, and 20% glycerol) and kept at -70°C until assay. Reactions (2 ml) contained (final concentration) 40 mM HEPES buffer (pH 7.5), 8% sucrose, 0.8% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, (CHAPS), 10 mM DTT, and 1 mM substrate (DEVD-AFC; Kamiya Biomedical, Thousand Oaks, CA) plus tissue extract (100 μl). Fluorescence changes (excitation 400 nm, emission 505 nm) were determined within the linear portion of the reaction at 5 min and 20 min of reaction at 37°C. Blanks containing all components except tissue extract were subtracted from the reactions. A standard curve of fluorescence vs. free 7-amino-4-trifluoromethyl-coumarin (Sigma) was used to calculate the specific activity of caspase-3-like enzymes in each sample. Protein concentration was mea-

sured by using a micro-Lowery assay kit. Activity was expressed as picomoles per minute per milligram protein.

Treatment Groups for the Study of Organ Dysfunction and Blood Chemistry

The following groups of animals were studied to examine the effects of pharmacological inhibition of PARP on the rise in ileum permeability, release of liver enzymes, and development of metabolic acidosis: 1) pretreatment with PARP inhibitor 3-AB given intravenously at 15 min before the initiation of hemorrhage (20 mg/kg) and at the initiation of resuscitation (10 mg/kg); 2) pretreatment with the structural analog 3-aminobenzoic acid (3-ABA) given intravenously at 15 min before the initiation of hemorrhage (20 mg/kg) and at the initiation of resuscitation (10 mg/kg); 3) posttreatment with PARP inhibitor 3-AB given intravenously as a bolus at 5 min before the initiation of resuscitation (20 mg/kg); and 4) sham-treated animals were time matched, but did not receive hemorrhage or resuscitation procedures.

The doses of 3-AB employed in the present studies are comparable with the doses used in previous *in vivo* studies of hemorrhagic shock (21, 30, 31) and in other models of injury (6, 9, 10, 26, 31, 34, 38, 39, 44, 49). 3-AB also has the capacity to act as an antioxidant at higher concentrations (35). Therefore, one group of animals received the same dose of 3-ABA, which served as an antioxidant control.

Measurements of Organ Dysfunction

Ileum permeability. Measurements of ileum permeability were made between 60 and 90 min of resuscitation. The procedure for assessing ileum permeability was modified from a previously published method (23). Animals were anesthetized with isoflurane (1–2%, balance oxygen), and a laparotomy was performed to allow access to the intestines. Holes were placed at both ends of a 10-cm segment of the ileum proximal to the cecum, and the contents were gently removed by irrigation with 10 ml Ringer solution. One end of the ileum was ligated, and the ileum was filled with 1 ml of FITC-dextran solution (25 mg/ml in 0.1 M PBS, pH 7.2). The other end of the ileum was then ligated so as not to disturb circulation through the major branches of mesenteric circulation to the segment of ileum. The intestines were returned to the abdominal cavity, and the abdomen was sutured. The animals remained on a temperature-controlled heating pad during this measurement. Thirty minutes later, the abdomen was opened and a 1 ml sample of hepatic portal vein blood was removed with an 18-gauge angiocath and syringe. An aliquot (0.1 ml) of whole blood was added to 1.9 ml of fluorescence buffer (50 mM Tris, pH 10.3, containing 150 mM NaCl). The diluted sample was centrifuged (3,000 *g* for 7 min at 4°C), and the fluorescence of the supernatant was determined with a fluorometer (Perkin-Elmer model L850B, set at 480 nm excitation and 520 nm emission). The original FITC-dextran solution was diluted, and aliquots were added to the fluorescence buffer to obtain a standard curve. This curve was used to quantify the levels of FITC-dextran observed in blood samples.

Blood chemistry and measurement of liver enzymes. Arterial blood samples (0.5 ml) were obtained in a heparinized syringe at the end of the baseline period, at the end of the period of hemorrhagic shock, and at 60 min after the start of resuscitation. These samples were analyzed for blood gases, pH, systemic base excess (SBEc model ABL 520; Radiometer, Westlake, OH), or lactic acid (YSI model 2700 Stat Select analyzer; Yellow Springs Instruments, Yellow Springs, OH). A sample of arterial blood (0.5 ml) was obtained without

heparinization 60 min after the start of resuscitation. This sample was centrifuged at 3,000 *g* (4°C), and the serum was analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as markers of liver injury with an automated analyzer (VetTest model 8008; IDEXX, Westbrook, ME).

Statistics

Sequential measurements of MAP in the same animals (Fig. 1) were compared with repeated-measures ANOVA on ranks. Individual time points were compared with baseline values by using Dunn's method. Comparisons of PARP activity in ileum isolated at different times (Fig. 2) were made with one-way ANOVA followed by the Student-Newman-Keuls method since these were independent samples. The effect of 3-AB on PARP activity was tested with paired *t*-tests within each experimental period. Activity of caspase (Fig. 3) in sham-treated and 60-min resuscitated animals was compared by *t*-test. Treatment effects on ileum permeability (Fig. 4), liver enzymes (Fig. 5), and blood chemistry (Fig. 6) were tested by using one-way ANOVA followed by the Student-Newman-Keuls method. Values are presented as means \pm SE with significance accepted at $P < 0.05$.

RESULTS

Effects of Hemorrhage and Resuscitation

MAP decreased from 124.9 ± 1.8 mmHg at baseline to 39.8 ± 0.8 mmHg at the end of the 60-min hemorrhage period (Fig. 1). The volume of blood shed during the hemorrhage period was 15.49 ± 0.55 ml (3.87 ± 0.10 ml/100 g body wt) in these animals. This shed volume includes the blood sample volume (0.5 ml) taken for blood chemistry measurements at the end of the baseline period. The volume of Ringer solution given toward the end of the hemorrhage period to maintain the hemorrhage pressure near 40 mmHg was

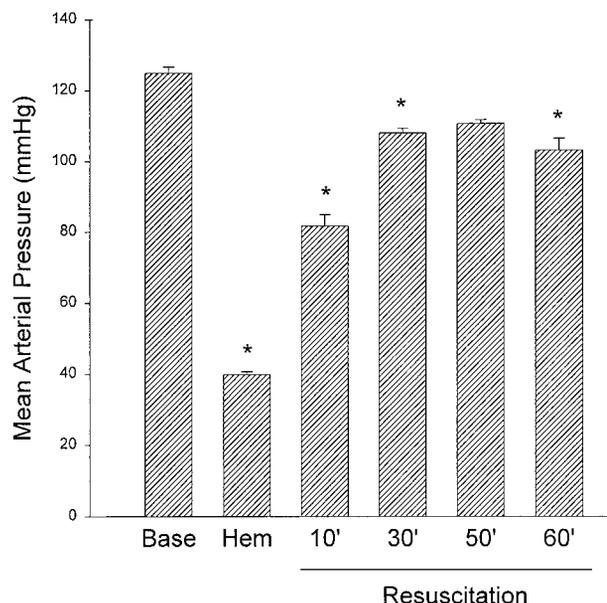


Fig. 1. Mean arterial pressure values observed in animals at baseline before hemorrhage (Base), at the end of the hemorrhage period (Hem), and at the end of each phase of the resuscitation. Values are means \pm SE; $n = 17$. *Significantly different from baseline.

2.38 ± 0.73 ml (0.56 ± 0.16 ml/100g body wt) in these animals. Resuscitation was accomplished with a combination of Ringer solution and PRBC. Blood pressure increased with the infusion of Ringer solution (10-min value), and further increase was observed following infusion of PRBC (30-min value). Blood pressure was maintained well above 100 mmHg through the end of the experimental period, suggesting that there was not severe underresuscitation. Hemorrhagic shock caused significant acidosis (pH 7.09 ± 0.04 vs. 7.43 ± 0.01), accumulation of lactic acid (16.41 ± 0.48 vs. 0.89 ± 0.06 mmol/l), base deficit (-22.44 ± 1.22 mmol/l vs. 0.98 ± 0.52), and hyperglycemia (411 ± 38 vs. 107 ± 7 mg/dl) compared with sham-treated animals, respectively. These values were partially restored toward baseline values 60 min after resuscitation began. Final values of hematocrit were 35.3 ± 3.6% at the end of the resuscitation period.

Activation of PARP

Ileum samples were isolated from animals at the end of each phase of the protocol. Sham-treated animals exhibited low PARP activity in the ileum (Fig. 2). PARP activity increased significantly (3.6-fold) in ileum samples isolated at the end of the hemorrhage period and was elevated fivefold following the initial 10 min of resuscitation with Ringer solution. Thus PARP activity was stimulated during the hemorrhage period and the initial period of resuscitation in this model.

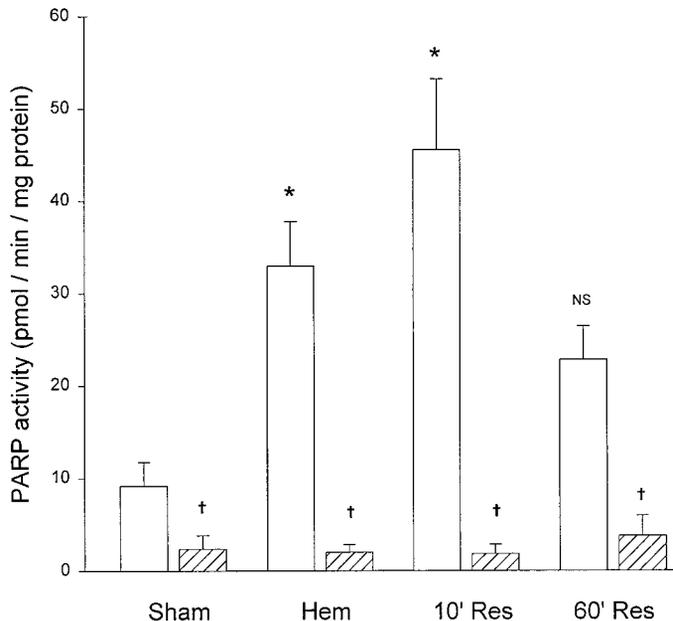


Fig. 2. Poly(ADP-ribose) (PARP) activity determined in ileum. Samples were isolated from sham-treated animals or from animals at the end of the hemorrhage period (Hem), following 10 min of resuscitation with Ringer solution (10' Res), or following resuscitation with Ringer solution, packed red blood cells, and additional Ringer solution (60' Res). Hatched bars are values determined with the addition of 12 mM 3-aminobenzamide (3-AB) in the assay mixture. Values are means ± SE; $n = 6-8$ at each time point. *Significantly different from sham-treated animals. NS, not significantly different from sham-treated animals. †Significantly different from the comparable uninhibited reactions.

PARP activity was significantly inhibited by the addition of 3-AB in all reactions. PARP activity, expressed per gram wet weight, was 325 pmol·min⁻¹·g wet wt⁻¹ at baseline, and peak levels were 1,528 pmol·min⁻¹·g wet wt⁻¹.

Similar measurements of PARP activity were attempted in liver samples. The activity was not significantly higher in livers isolated after 10 min of resuscitation than in livers isolated from sham-treated animals (65.5 ± 13.7 vs. 45.0 ± 7.3 pmol·min⁻¹·mg⁻¹ protein, respectively; $P = 0.270$). PARP activity measured in the sham-treated livers was significantly higher than in the sham-treated ileum samples (9.2 ± 2.6 pmol·min⁻¹·mg⁻¹) but was similar to the activity observed in the ileum samples isolated after 10 min of resuscitation (45.5 ± 7.6 pmol·min⁻¹·mg⁻¹). It is possible that PARP activity may have been stimulated during the preparation of the tissue extracts, producing high levels of PARP activity in liver from sham-treated animals. Liver tissue has a Ca²⁺/Mg²⁺ dependent endonuclease present in the nucleus that actively cleaves DNA (48) and may have caused nonspecific activation of PARP during the tissue preparation.

Analysis of PARP Cleavage in Ileum

The observation that PARP activity decreased in the ileum after 60 min of resuscitation compared with PARP activity observed after 10 min of resuscitation suggested that PARP may be cleaved by caspase activation. Therefore, we assessed PARP cleavage by Western blotting and directly measured caspase enzyme activity. Western blots of PARP protein in nuclei isolated from ileum samples (Fig. 3A) indicated that there was no disappearance of the 116-kDa active form of PARP and no significant appearance of the 85-kDa breakdown fragment in the sham-treated or the shocked animals. A positive control for the decrease in 116-kDa fragment and appearance of the 85-kDa fragment is also shown to indicate that the technique does detect PARP cleavage when it exists. Human cells were exposed to cyclohexamide (25 μg/ml) for 24 h in culture to induce apoptosis for comparison with control cells. Measurement of caspase-3 activity in the sham-treated and the shocked animals indicated that caspase-3 activity was not elevated at this time of resuscitation (Fig. 3B).

Organ Dysfunction

Dextran was infused into the ileum after 60 min of resuscitation, and the hepatic portal blood samples were taken after 90 min of resuscitation to measure ileum permeability (Fig. 4). The level of dextran was elevated 10-fold in the hepatic portal blood of animals pretreated with 3-ABA compared with the level observed in sham-treated animals. Pretreatment with the PARP inhibitor 3-AB resulted in a significant inhibition of the rise in ileum permeability. In contrast with the pretreatment mode of PARP inhibition, the rise in ileum permeability was not prevented with the

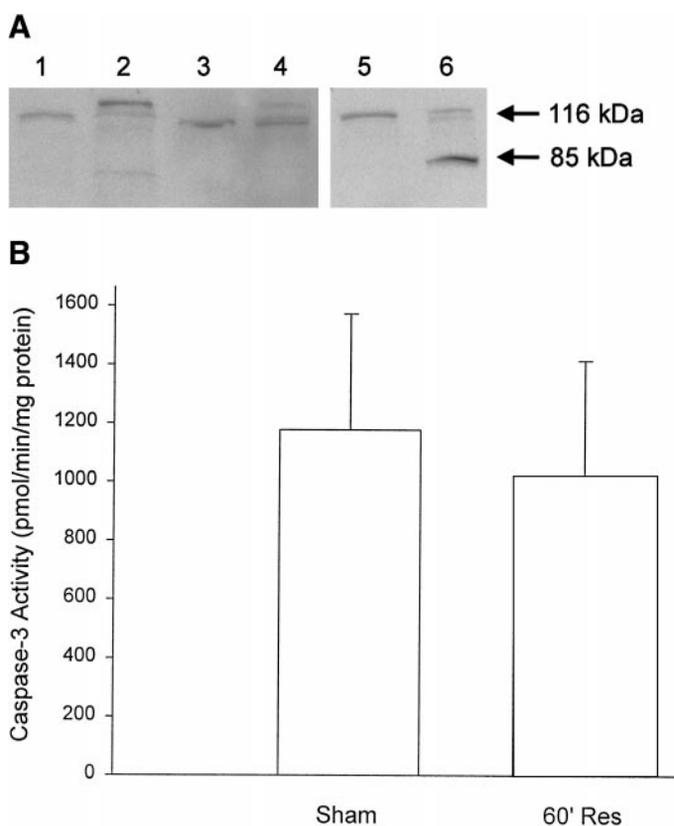


Fig. 3. Analysis of PARP breakdown by caspases. *A*: Western blot showing PARP protein in nuclear samples isolated from ileum samples of sham-treated animals (*lanes 1 and 2*) and animals subjected to hemorrhage + 60 min of resuscitation (*lanes 3 and 4*). Positive control for cleavage of PARP from human cells in culture that were treated with cyclohexamide (*lane 5*) can be compared with control cells (*lane 6*). *B*: caspase-3 activity determined in extracts of ileum from sham-treated animals and animals receiving hemorrhage + 60 min of resuscitation.

addition of a bolus of 3-AB given 5 min before resuscitation (Fig. 4).

Measurements of liver transferase enzyme activity were made in arterial samples taken at the end of the 60-min resuscitation period. Arterial ALT values increased 6.5-fold in animals subjected to hemorrhage and resuscitation with 3-ABA treatment compared with the sham animals (Fig. 5A). Pretreatment with the PARP inhibitor 3-AB significantly reduced the values of arterial ALT compared with animals receiving 3-ABA pretreatment. There was no significant difference between animals that received 3-ABA pretreatment or 3-AB posttreatment. A similar pattern of liver injury was observed with arterial AST values (Fig. 5B). This suggests that liver injury was reduced by pretreatment with the PARP inhibitor 3-AB but that pretreatment with 3-ABA or posttreatment with a bolus of 3-AB showed evidence of liver injury.

Effects of PARP Inhibition on Acid-Base Status

Sham-treated animals had normal values of pH (Fig. 6A), arterial lactate (Fig. 6B), and arterial base deficit (Fig. 6C). Baseline measurements indicated that blood

chemistry was unchanged by pretreatment of the animals with 3-AB or 3-ABA (data not shown). Animals treated with 3-ABA showed significant acidosis (pH = 7.027 ± 0.030), lactic acid accumulation (17.66 ± 1.074 mmol/l), and base deficit (-24.76 ± 1.22 mmol/l) during the hemorrhagic shock period (Fig. 6). Pretreatment with 3-AB resulted in a smaller decrease in arterial pH, less lactic acid accumulation, and a lower base deficit compared with animals pretreated with 3-ABA. This finding suggests that inhibition of PARP before the induction of hemorrhagic shock reduced the severity of metabolic acidosis produced by hemorrhagic shock. A similar pattern persisted throughout the resuscitation period. When 3-AB was given as a bolus 5 min before resuscitation, the metabolic acidosis was not inhibited during hemorrhage or resuscitation (Fig. 6).

DISCUSSION

This study demonstrates that PARP enzyme activity is elevated in the ileum by hemorrhage alone and during early resuscitation following hemorrhagic shock. Inhibition of PARP activity by pretreatment with 3-AB lessened the rise in permeability of the ileum and reduced the appearance of liver enzymes in the plasma observed after hemorrhage and resuscitation. In addition, the inhibition of PARP slowed metabolic acidosis, lactate accumulation, and base deficit observed during hemorrhagic shock and subsequent resuscitation. In contrast, posttreatment with 3-AB,

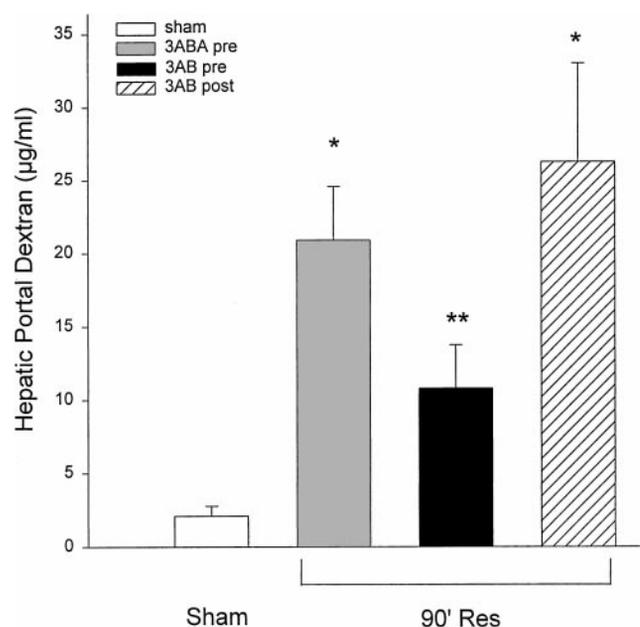


Fig. 4. Measurements of ileum permeability made in sham-treated rats or in rats that were pretreated with 3-aminobenzoic acid (3-ABA) or were pretreated or posttreated with 3-AB. Dextran was infused in the lumen of the ileum at 60 min of resuscitation, and the hepatic portal sample was taken at 90 min of resuscitation. Values are means \pm SE; $n = 5-6$ in each group. *Significantly different from sham-treated animals (but not from each other). **Significantly less than the animals receiving 3-ABA pretreatment or 3-AB posttreatment.

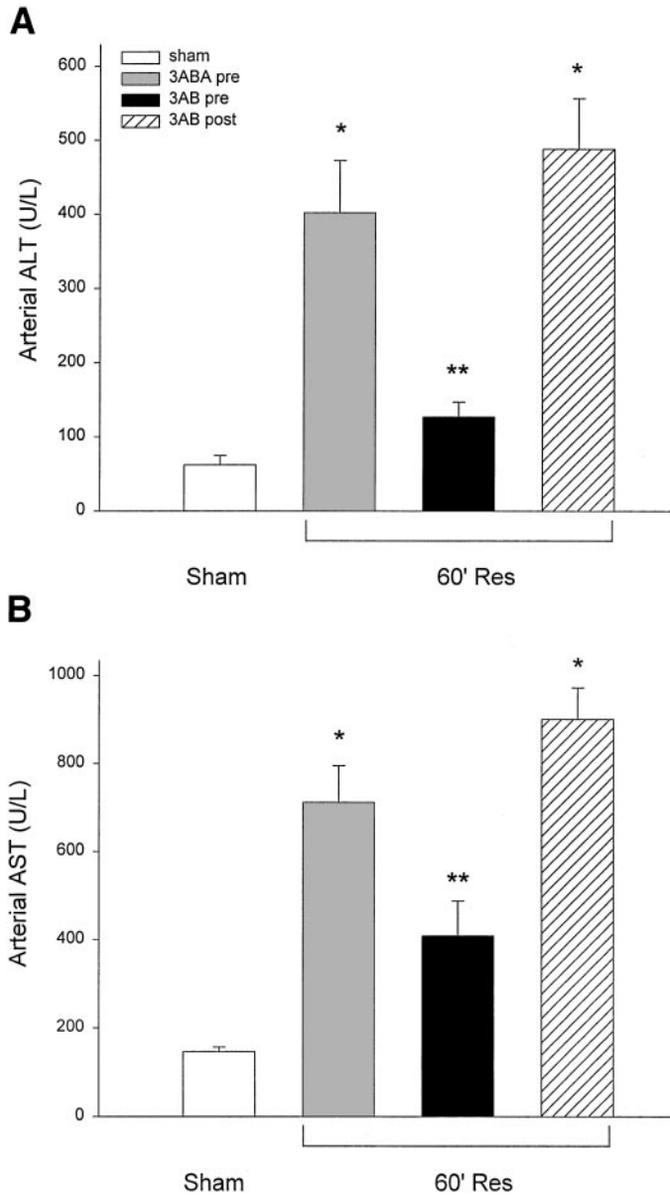


Fig. 5. Values of arterial alanine aminotransferase (ALT) (A) or arterial aspartate aminotransferase (AST) (B) determined at the end of the 60-min resuscitation period. Measurements were made in sham-treated animals or in rats that were pretreated with 3-ABA or were pretreated or posttreated with 3-AB. Values are means \pm SE; $n = 5-13$ in each group. *Significantly different from sham-treated animals (but not from each other). **Significantly less than the animals receiving 3-ABA pretreatment or 3-AB posttreatment.

given as a bolus 5 min before resuscitation, was ineffective in reducing the rise in ileum permeability, the appearance of liver enzymes, and the development of indices of metabolic acidosis in this model. These findings suggest a role of PARP activation in the pathogenesis of organ injury and metabolism during hemorrhagic shock.

Hemorrhagic shock causes mesenteric constriction (40). Intense vasoconstriction induces tissue hypoxia in the ileum that can lead to depletion of tissue ATP levels and enhanced intestinal epithelial permeability (43). Disturbances in the mucosal barrier of the ileum

may play a role in the induction of sepsis in trauma and shock via the translocation of bacteria or bacterial products (13). Therefore, it is important to consider the link between excessive PARP activation and intestinal permeability. Increased intestinal permeability associated with ischemia and reperfusion is reduced by PARP inhibition (10, 20) or by PARP knockout in mice (20); however, this relationship has not previously been tested in hemorrhage and resuscitation. The present

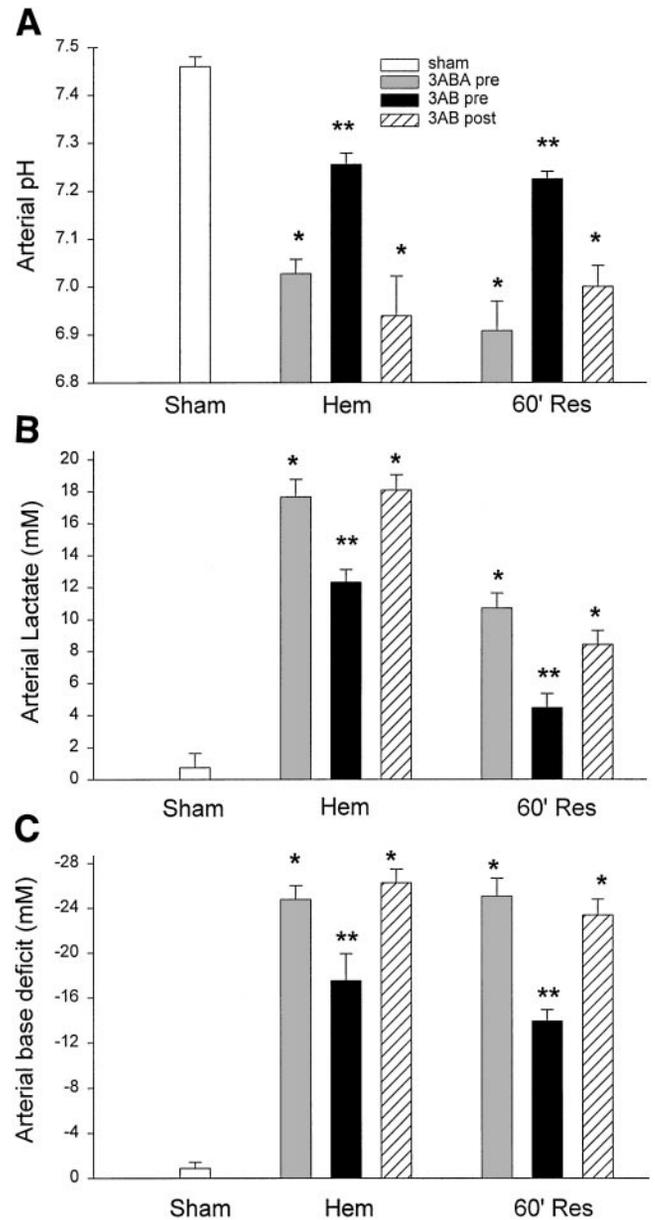


Fig. 6. Values of arterial pH (A), lactate (B), and base deficit (C) measured in animals from the different treatment groups. Treatments included sham treatment, pretreatment with 3-ABA, pretreatment with 3-AB, or posttreatment with 3-AB. Measurements were made at the end of hemorrhage (Hem), after 60 min of resuscitation, or in time-matched sham-treated animals. Values are means \pm SE; $n = 6-8$ in each group. *Significantly greater than sham-treated values but not significantly different from each other within a time point. **Significantly different from the groups receiving 3-ABA pretreatment or 3-AB posttreatment.

data indicate that PARP is activated in the ileum as a response to hemorrhage and to hemorrhage with resuscitation and that the inhibition of PARP activity provides protection against the dysfunction of this organ.

PARP activation occurs during hemorrhagic shock in the present studies, suggesting that DNA damage occurs before resuscitation. Severe hemorrhagic shock causes ischemia in the gut. It is clear that reperfusion of ischemic tissue leads to the formation of oxygen radicals (10, 14). In the classic paradigm, resuscitation would be required to stimulate reactive oxygen species to produce DNA damage, which is required for PARP activation. Because PARP activity was stimulated during the hemorrhage period with no resuscitation (Fig. 2), it is important to consider that free radical-mediated DNA injury occurred in the ileum during the period of reduced flow without resuscitation. The respiratory chain is one of the major sources of free radicals in cells (24). Becker and colleagues (1, 41, 42) have recently examined electron leak from the electron transport system during anoxia in cardiomyocytes. These studies indicate that leak of electrons occurs between NADH DH in complex I and the ubisemiquinone site with cytochrome *b-c1* segment of complex III when the electron transport system is reduced and some oxygen is present. This occurs even at the low oxygen concentrations observed during ischemia. In addition, low concentrations of arginine cause nitric oxide synthase to form superoxide (45–47) and acidic conditions can cause nonenzymatic formation of nitric oxide (51, 52). This could lead to the formation of peroxynitrite that may damage the DNA. It is unclear whether these conditions contribute to the injury developed in vivo. Recent data show that occlusion of the superior mesenteric artery with no reperfusion produced fragmentation of DNA after 15 min (5% of DNA) and 60 min (12% of DNA) (25). This fragmentation was increased by reperfusion at either time, but the data illustrate that DNA fragmentation can occur in the ileum during low-flow states. Alternatively, there could be intermittent perfusion of the ileum during the hypotensive phase of the experiment, since it is unlikely that there is complete cessation of flow during hemorrhage (40).

The finding that PARP is activated during the hypotensive phase of shock suggests that pretreatment with PARP inhibitors may be required to confer protection. Many studies of PARP inhibitors in hemorrhagic shock have employed models in which the PARP inhibitor was added before the induction of hemorrhagic shock (31, 33). The use of knockout mice also provides a pretreatment absence of PARP-1 activity (19). We have previously observed protection of aortic constrictor responses following the addition of 3-AB at the onset of resuscitation from hemorrhagic shock (30). McDonald et al. (21, 22) also observed a reduction in the appearance of liver, kidney, and pancreatic enzymes in blood when PARP inhibitors were added 5 min before resuscitation and infusion was continued throughout the resuscitation. Two possible explanations may account

for the differences in the success of posttreatment with PARP inhibitors following hemorrhagic shock. First, these models employed anesthetized rats and the hemorrhagic shock was probably less severe than the conscious model of hemorrhage used in the present studies (40 mmHg, 3.87 ml blood shed/100 g body wt). Second, the posttreatment in the present study was given as a bolus infusion before resuscitation and was not continued throughout the resuscitation period. Future studies should compare models of different severity and the effects of continual infusion to determine conditions in which posttreatment with PARP inhibitors provides benefits. This approach would further evaluate the clinical application of PARP inhibitors in improving the outcome of resuscitation following hemorrhagic shock.

The increase in PARP activity detected in the present studies of the ileum (5-fold) was similar to the increase observed in organs and isolated cells subjected to a variety of insults (8, 11, 15, 18, 22, 26, 29, 36, 37). A study that specifically measured PARP activity in intestinal mucosal cells found a doubling of PARP activity following splanchnic artery occlusion and reperfusion (10, 20). Technical differences in the preparation of the enzyme for analysis may account for some of the differences in PARP activation of the ileum. For instance, the studies of ischemia and reperfusion of the intestine used permeabilized cells (10), whereas the present studies used nuclei that were permeabilized following their isolation from tissue homogenates.

The dose of 3-AB administered in the present study is comparable with doses used in previous studies of hemorrhagic shock (21, 30, 31) and in other in vivo models of injury (6, 9, 10, 26, 31, 34, 38, 39, 44, 49). At high concentrations, 3-AB also has the capacity to act as an antioxidant (35), which is a possible mode of action that may complicate the interpretation of the data while evaluating the role of PARP in studies employing this agent. The present studies also included a treatment group that received 3-ABA, a compound that has the same benzene ring structure but lacks the amino group and therefore does not inhibit PARP. Therefore, potential antioxidant mechanisms of protection should be observed in this group. Since 3-ABA did not protect against the rise in ileum permeability (Fig. 4), liver ALT, or AST enzymes in blood (Fig. 5), it appears that the protective effects of 3-AB are not due to antioxidant properties. The increase in liver enzymes was attenuated by the addition of 0.03 and 0.3 mg/kg of a water-soluble isoquinolone derivative in another study of hemorrhagic shock in anesthetized rats (22), suggesting that more potent inhibitors may provide beneficial effects at lower doses.

The present studies show that activation of PARP occurs in the ileum at the end of hemorrhagic shock without resuscitation and during the early part of resuscitation. The decline in PARP activity observed following 60 min of resuscitation is not due to cleavage by caspase-3. Inhibition of PARP reduces metabolic acidosis observed during hemorrhage and during re-

suscitation and reduces the rise in ileum permeability and the release of liver enzymes observed after resuscitation. Thus PARP activation, occurring during hemorrhage and during the early phases of resuscitation, contributes to organ dysfunction. The addition of the PARP inhibitor as a bolus just before resuscitation was ineffective in the present model, suggesting that this approach to therapy may require early treatment or may require subsequent infusion of PARP inhibitor throughout resuscitation.

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