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Sandro B. Rizoli, Andras Kapus, Jie Fan, Yue H. Li, John C. Marshall, and Ori D. Rotstein

Hypertonic resuscitation fluids are known to be effective in restoring circulating volume in the hypovolemic trauma patient. Previous studies have suggested that hypertonicity might exert effects on immune cells leading to an altered host response. The present studies evaluated the effect of hypertonic resuscitation on the development of lung injury in a hemorrhagic shock model in which antecedent shock primes for increased lung neutrophil sequestration in response to intratracheal LPS. Resuscitation with hypertonic saline significantly reduced albumin leak, bronchoalveolar lavage fluid neutrophil counts, and the degree of histopathologic injury compared with resuscitation with Ringer’s lactate. Both in vivo and in vitro data suggest that this beneficial effect may be related to altered adhesion molecule expression by the neutrophil. Specifically, hypertonicity induced shedding of L-selectin and prevented LPS-stimulated expression and activation of CD11b, both of which might contribute to reduced sequestration in the lung. Impaired up-regulation of lung ICAM-1 may have also participated, although ex vivo studies suggest that alterations in neutrophils were sufficient to account for the effect. Lung cytokine-induced neutrophil chemoattractant did not differ between animals resuscitated with hypertonic saline vs Ringer’s lactate. Considered together, these studies demonstrate a possible novel approach to inhibiting organ injury in disease processes characterized by neutrophil-mediated damage. The Journal of Immunology, 1998, 161: 6288–6296.

The major objective of fluid resuscitation in the traumatized patient with hemorrhagic shock is the restoration of effective circulating intravascular volume. The use of crystalloid solutions for this purpose is generally considered to be optimal, given their effectiveness, low cost, and minimal morbidity. A meta-analysis of studies comparing the use of crystalloid to colloid solutions in patients with hypovolemic shock demonstrated a 12% reduction in overall mortality when patients were resuscitated with a crystalloid-based approach (1). Resuscitation with small volumes of hypertonic saline (HTS) solutions has been shown to restore circulating volume and hemodynamics, in part through imbiberment of intracellular fluid into the vascular space (1–4). The ability to resuscitate with reduced fluid volume has been considered advantageous, in that it might reduce the potential consequences of third space fluid sequestration, such as the development of cerebral edema in the head-injured patient or pulmonary edema in the patient with contused lung (3, 5). Further, some studies have reported improved cardiac contractility with HTS resuscitation regimens (6). Both experimental and clinical trials have suggested a more favorable outcome following HTS resuscitation from hemorrhagic shock (3, 4, 6), although the mechanisms underlying this perceived benefit remain poorly elucidated.

Recent studies have suggested that hyperosmolar solutions exert alterations in immune cell function that may contribute to improved outcome (7–9). Junger and colleagues reported the ability of HTS resuscitation to reverse hemorrhage-induced suppression of splenocyte proliferation in a murine model (8). In subsequent experiments, these investigators demonstrated that hyperosmolarity can enhance mitogen-stimulated T cell proliferation in vitro through a mechanism involving tyrosine phosphorylation and activation of the p38 mitogen-activated protein kinase. The reversal of trauma-induced immunosuppression by HTS may represent a mechanism by which this resuscitation strategy may lessen septic complications and thus enhance survival.

Significant morbidity in the trauma patient also results from the development of multiple organ dysfunction, even in the absence of infection (10). This process is predominantly mediated by the sequestration of activated neutrophils within the microvasculature and subsequent release of their injurious products, such as reactive oxygen species and proteolytic enzymes. The lung appears to be a particular target, with the appearance of the acute respiratory distress syndrome in up to 40% of the traumatized patient population (10, 11). Hemorrhagic shock is believed to contribute to the pathogenesis of acute respiratory distress syndrome by rendering the patient more susceptible to a second, seemingly trivial, inflammatory stimulus, the so-called two-hit model (12, 13). In this regard, previous studies have suggested that the process of ischemia-reperfusion might mediate this effect by priming circulating neutrophils for increased superoxide production, and therefore enhanced cytotoxicity, once they are sequestered in the lung (12, 13).

Previous reports have documented the ability of hyperosmolar solutions to inhibit a variety of neutrophil functions, including the generation of reactive oxygen species (14, 15). We therefore hypothesized that HTS resuscitation from hemorrhagic shock might...
lessen the development of subsequent lung injury. To test this possibility, we used a rodent model in which resuscitated hemorrhagic shock primes for increased pulmonary leukosequestration and albumin leak in response to a small dose of intratracheal LPS (16). The data demonstrate that HTS resuscitation from shock markedly reduced lung injury in response to LPS by preventing neutrophil sequestration. This appeared to be predominantly mediated via an effect on neutrophil adhesion molecule expression.

Materials and Methods

Materials

Ficoll and dextran T500 were purchased from Pharmacia Biotech (Baie D’Urfe, Canada). Trizma (Tris base), 2-ME, FMLP, BSA, PMA, citrate, LPS (Escherichia coli O111: B4), propidium iodide, sarcosyl, and guanidine isothiocyanate were obtained from Sigma (St. Louis, MO). [125I]albumin was purchased from Fratran-Kirkland, Canada), EDTA from BDH (Toronto, Canada), Triton X-100 from Caledon (Georgetown, Canada), and 51Cr from Amersham (Oakville, Canada). Cell Wells tissue culture chambers were from Corning Costar (Cambridge, MA). Glyceraldehyde-3-phosphate dehydrogenase G3PDH was obtained from Clontech (Palo Alto, CA), gelatin from Bio-Rad (Hercules, CA), dls-C- (5) from Molecular Probes (Eugene, OR), and human fibronectin from Boehringer Mannheim (Laval, Canada). Human recombinant TNF-α, valinomycin, and gramicidin were purchased from Calbiochem (La Jolla, CA). The anesthetic drugs used were pentobarbital from MTC Pharmaceuticals (Cambridge, Canada), ketamine from Ragan/STB (London, Canada), and xylose from Bayer (Etobicoke, Canada). The rat neutrophil isolating kit NIM2 was purchased from Cardinal Associates (Santa Fe, NM). All chemicals used were of the highest purity available.

Solutions

DMEM, HBSS, medium 199, and PBS were obtained from Life Technologies (Burlington, Canada). Ringer’s lactate (RL) was obtained from Baxter (Toronto, Canada). and 10% buffered formalin was purchased from Fisher Chemical (Fairlawn, NJ). PMNs were isolated in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT) and penicillin/streptomycin (Life Technologies). TBS contained 100 mM NaCl and 50 mM Tris, pH 7.4. Isotonic NaCl solution contained 3 mM KC1, 5 mM glucose, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES, pH 7.4. Hypertonic medium was obtained by adding extra NaCl to the DMEM containing 10% FCS. The Toronto Hospital Pharmacy Department prepared HTS/7.5% NaCl for use in resuscitation of animals.

Abs and cell line

All Abs were monoclonal. The following were anti-human: FITC-labeled anti-L-selectin (Dreg 56 from ImmuneTech (Marseille, France) and anti-CD11b (Leu 1) phycocerythrin from Becton Dickinson (San Jose, CA). The following were anti-rat: FITC-labeled anti-L-selectin HRL2 from D’Urfe, Canada). ECV304 (HUVEC line) was obtained from American Type Culture Collection (Manassas, VA).

Animal model

Adult male Sprague-Dawley rats, weighing 300–350 g (Charles River, St. Constant, Canada), were anesthetized with i.p. ketamine (80 mg/kg) and xylazine (8 mg/kg). The right carotid artery was cannulated with a 22-gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling, and resuscitation. Hemorrhagic shock was induced by blood withdrawal and reduction of the MAP to 40 mm Hg within 15 min. This blood pressure was maintained by further blood withdrawal if the MAP was >45 mm Hg, and by infusion of 0.5 ml of RL if the MAP was <35 mm Hg. Shed blood was collected into 0.1 ml of citrate/m/111 blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood and either an equivalent amount of RL or a 4 ml/kg volume of 7.5% NaCl (HTS) over a period of 2 h. The catheter was then removed, the carotid artery was ligated, and the cervical incision was closed.

One hour later, 100 μg of LPS in 200 ml of saline was administered intratracheally through a 14-gauge angiocath tracheotomy. Sham animals were instrumented but not bled, and saline alone was instilled intratracheally. Animals were sacrificed by pentobarbital overdose at various time points depending on the specific study. Blood samples were collected during the experiment for serum osmolality. Serum was separated by centrifugation, and osmolality was measured in an automated osmometer Advanced Osmometer 3D3 (Two Technology Way, Norwood, MA). For histologic assessment, the whole lungs were fixed in 10% neutral buffered formalin (pH 7.4) and later stained with hematoxylin/eosin and examined using an optical microscope.

Bronchoalveolar lavage (BAL)

BAL cell counts were determined at 4 h following LPS or saline intratracheal instillation. Immediately after sacriﬁce, the lungs were perfused via the tracheotomy cannula with cold PBS/0.1 mM EDTA in 10-ml aliquots and were gently withdrawn to a total of 40 ml. For cell counts and differential, BAL fluid was centrifuged at 300 × g for 10 min. After discarding the supernatant, the pelleted cells were resuspended in serum-free DMEM. Total cell counts were made on a grid hemocytometer, and the differential cell counts were made on a cytopsin-prepared slide stained with Wright-Giemsa. The number of neutrophils was calculated as follows: number of neutrophils = total cell count × % of neutrophils in BAL fluid sample.

Myeloperoxidase (MPO) assay

Lung samples were thawed, and approximately 0.5 g of tissue was homogenized in 25 ml of potassium phosphate (10 mM; pH 7.4) for 1 min using a Brinkmann Polytron (model PT10/35, Brinkmann Instruments, Westbury NY). The homogenate was centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was discarded, and the pellet was resuspended and homogenized in 25 ml of potassium phosphate (50 mM; pH 6.0) containing 0.5% HTAB. The homogenate was frozen overnight at −70°C, rehomogenized for 1 min, and sonicated (model VC 50T, Sonics & Materials, Danbury, CT) at 40 W for 1 min. After centrifugation as described above, the supernatant was collected and total of 500 cells were counted in each sample, and the number of neutrophils was calculated as follows: number of neutrophils = total cell count × % of neutrophils in BAL fluid sample.

Transpulmonary albumin flux

Rats were injected with 1 μCi of [125I]albumin into the tail vein immediately preceding intratracheal LPS or saline. Six hours later, both 1 ml of blood collected by cardiac puncture and three samples of 1 ml of BAL fluid were analyzed by a gamma spectrometer. The [125I]albumin leak was corrected to blood counts per minute and expressed as the transpulmonary albumin leak index as follows: transpulmonary albumin leak index = (cpm/ml in BAL fluid)/(blood cpm/ml).

Neutrophil isolation and labeling

Human neutrophils. Cells were isolated from fresh blood drawn by venipuncture into heparinized tubes. Isolation was performed using 3% dextran sedimentation and centrifugation on ficoll-Hypaque cushions as described previously (18). After erythrocyte lysis in 0.84% NH4Cl, the PMNs were resuspended in isotonic medium or hypertonic medium (350 mOsmol/kg H2O) and were gently withdrawn to a total of 40 ml. For cell counts and differential, BAL fluid was centrifuged at 300 × g for 45 min) the pelleted cells were washed in HBSS twice and incubated in either isotonic medium or hypertonic medium (350 mosmol) for 4 h. In some studies, freshly isolated cells were used immediately following the labeling step. Cells were pelleted by centrifugation at 300 × g and were resuspended in isotonic DMEM. An aliquot of cells from each group was counted to ensure equivalent labeling. (1480 Wizard 3 automatic gamma counter, Wallac, Turku, Finland). There was no difference in the labeling between isotonic and hypertonic treatments.
Detection of radiolabeled PMN in the lung

In some studies, we examined whether isolated rat neutrophils exposed to isotonic or hypertonic medium ex vivo might exhibit differential sequestration in the lung. Rats underwent either sham manipulation or hemorrhagic shock with RL resuscitation as described. Following PMN isolation, ex vivo labeling, and incubation in isotonic or hypertonic solution as described above, 2 × 10^6 51Cr-labeled isotonic medium or hypertonic medium, resuspended in isotonic medium, was injected into the tail vein of the rats immediately before intratracheal LPS or saline instillation. At sacrifice 4 h later, 1 ml of blood was collected by cardiac puncture. The lungs were harvested after being flushed with PBS/EDTA through a cannula inserted in the pulmonary veins until the effluent was completely clear. 51Cr-labeled PMN accumulation in the lung was analyzed in the gamma spectrometer and calculated as follows: 51Cr-labeled PMN lung accumulation = (lung cpm/g tissue)/blood cpm/ml.

Assessment of neutrophil viability

At the end of the preparation phase, two approaches were used to assess PMN viability. By trypan blue exclusion technique, PMN viability exceeded 95% and did not differ between isotonic and hypertonic treatment. Measurement of transmembrane potential was also tested using the anionic fluorescent probe diS-3-C (5) as previously described (19).

Measurement of neutrophil surface CD11b and L-selectin

In vitro studies on human PMN. Flow cytometric analysis was used to evaluate surface expression of these adhesion molecules. Isolated human PMN were incubated in isotonic medium or hypertonic medium at 350 or 500 mosmol for up to 4 h. PMN were resuspended in isotonic medium and incubated with FITC-labeled anti-CD11b Ab or anti-CD11b Ab at a 1/10 dilution for 20 min at 4°C. PMNs were then analyzed on the FACScan (Becton Dickinson, Palo Alto, CA) using an FL1 detector (488 nm excitation wavelengths). Typically, 5,000 cells were analyzed per condition, and the results were expressed as the mean channel fluorescence. CD11b expression was measured in resting and stimulated human PMN. The following stimuli were used: LPS (1 μg/ml for 1 h), PMA (50 nM for 20 min), or FMLP (10^-7 M for 20 min).

Detection of surface markers on rat PMN in whole blood. For CD11b detection, 100 μl of whole rat blood was incubated with FITC-labeled anti-CD11b Ab at 1/10 dilution at room temperature for 20 min. Erythrocytes were lysed with lysing buffer (0.84% NH4Cl), the remaining cells washed twice with DMEM and analyzed on the FACScan as above. For measurement of L-selectin, PMNs were isolated from whole blood and incubated with FITC-labeled L-selectin at a 1/10 dilution for 20 min at 4°C. PMNs were then analyzed on the FACScan as described above.

Neutrophil adhesion assays

ECV304 human endothelial cells were grown to confluence on fibronectin-coated tissue culture chambers. To induce high expression of ICAM-1 (25) the ECV304 cells were stimulated with TNF-α (1 ng/ml) for 6 h, and subsequently washed with fresh DMEM and 10% FCS. Neutrophils were treated with iso- or hypertonic (350 mosmol) medium for 4 h, then incubated in the presence or the absence of LPS (1 μg/ml) for 1 h. Neutrophils were resuspended in isotonic DMEM and 10% FCS and layered on top of the prestimulated endothelial cells for 30 min at 37°C. Nonadherent neutrophils were removed by repeated washes with PBS. The number of adherent neutrophil was determined by microscopy by two investigators blinded to the manipulations. Three high-power fields were counted per well, and each group was plated in six different wells.

Northern blot analysis

Total RNA from lungs was obtained using the guanidium-isothiocyanate method (20). Briefly, lungs were harvested and homogenized in 10 ml of 4 M guanidine isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, and 100 mM β-ME. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel, and transferred to nylon membrane. Hybridization was conducted using a 32P-labeled random-primer murine ICAM-1 cDNA probe (American Type Culture Collection) and a 32P-labeled end-labeled 30-base oligonucleotide probe for cytokine-induced neutrophil chemoattractant (CINC) with the sequence 5′-GCG GCATCACCTTCAAACCTCTGAGCTTC-3′, which is complimentary to nucleotides 134–164 of CINC cDNA (21) provided by Dr. Timothy S. Blackwell (Vanderbilt University School of Medicine, Nashville, TN). Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens as previously reported (22). To control for loading, blots were then stripped and reprobed for G3PDH, a ubiquitously expressed housekeeping gene (23). Expression of mRNA was quantitated using a PhosphoImager and accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and was normalized to the G3PDH signal.

Western blot analysis

To study ICAM-1 protein expression in the lung, whole rat lungs were homogenized in TBS/1% Triton X-100 solution. Lung tissue homogenate samples were separated on a 15% SDS-PAGE under nonreducing conditions (24). Separated proteins were electroblotted onto polyvinylidene difluoride membrane and blocked for 1 h at room temperature with Tris-buffered saline containing 1% BSA. The membranes were then incubated with a 1/1000 dilution of antiserum against ICAM-1 at room temperature for 1 h. Ag-Ab complexes were identified with goat anti-rabbit IgG tagged with horseradish peroxidase (Sigma, St. Louis, MO) and detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

Statistical analysis

Data are presented as the mean ± SE of the number of experiments indicated. When blots are shown, they are representative of at least three independent studies. Significance was assessed using Student’s t test and one-way analysis of variance with post-hoc testing with the Tukey-Kramer multiple comparisons test. p < 0.05 was considered significant.

Results

Effect of hypertonic resuscitation on restoration of blood pressure and serum osmolarity following hemorrhagic shock

Animal survival at the end of the experimental period in both resuscitation groups exceeded 90%, with deaths due to tracheal obstruction secondary to the presence of the intratracheal cannula. HTS resuscitation effectively restored MAP to preshock levels (data not shown). Fig. 1 illustrates the serum osmolarity at various time points following resuscitation with HTS or RL. As shown, HTS caused a rapid increase in osmolarity that peaked at approximately 500 mosmol at 5 min and then returned to approximately 350 mosmol by 1 h. This was sustained over the ensuing 3 h.

HTS resuscitation reduces lung injury following LPS

Our laboratory has previously reported that resuscitated shock primes the lung for increased transpulmonary albumin leak in response to LPS (16). As demonstrated in Fig. 2, both shock alone and LPS alone caused small increases in albumin leak. The administration of LPS following shock resuscitated with RL caused a further significant rise in the albumin leak. However, when shocked animals were resuscitated with HTS, the increase in albumin leak was totally prevented.

Neutrophil sequestration is a hallmark of acute lung injury. BAL fluid was evaluated for neutrophil counts. Fig. 3A shows alveolar...
PMN counts under each of the conditions. Compared with shock/LPS animals resuscitated with RL, the use of HTS caused >50% reduction in the number of PMN recovered in the BAL. As a measure of overall lung sequestration of PMNs, lung MPO levels were studied. As demonstrated in Fig. 3A, BAL neutrophil counts. Four hours after intratracheal LPS, BAL fluid [125I]albumin was measured by gamma counting and normalized for blood counts as indicated in Materials and Methods. The data represent the mean ± SEM for five animals per group. *, p < 0.05 vs sham; **, p < 0.01 vs sham; ***, p < 0.01 vs shock/LPS/RL.

FIGURE 2. Transpulmonary albumin leak index following shock/resuscitation with either RL or HTS. Six hours after intratracheal LPS, BAL fluid [125I]albumin was measured by gamma counting and normalized for blood counts as indicated in Materials and Methods. The data represent the mean ± SEM for five animals per group. *, p < 0.05 vs sham; **, p < 0.01 vs sham; ***, p < 0.01 vs shock/LPS/RL.

Materials and Methods

Lung MPO. Lungs were harvested at 4 h after intratracheal LPS and evaluated for total MPO. Lungs were harvested at 4 h for six animals per group.

FIGURE 3. Neutrophil sequestration in the lungs following shock/resuscitation and LPS. A, BAL neutrophil counts. Four hours after intratracheal LPS, neutrophils in the BAL fluid were counted using a hemocytometer. The data represent the mean ± SEM for six animals per group. *, p < 0.01 vs sham; **, p < 0.01 vs shock/LPS/RL. B, Lung MPO. Lungs were harvested at 4 h following LPS and evaluated for total MPO as described in Materials and Methods. The data represent the mean ± SEM for three animals per group. *, p < 0.01 vs sham; **, p < 0.01 vs shock/LPS/RL.

Ex vivo treatment of PMN with hypertonicity prevents lung PMN sequestration

To discern whether the changes in PMN function were sufficient to account for the reduced pulmonary leukosequestration, PMNs were labeled ex vivo, exposed to isotonic or hypertonic medium for 4 h, and then resuspended in isotonic medium. They were then reinfused into sham animals or animals following RL resuscitation from shock, immediately before LPS administration (Fig. 6A). As expected, sham animals given isotonically treated PMN or hypertonicity treated PMN did not exhibit PMN accumulation in the lung. Further, shocked animals resuscitated with RL showed increased PMN counts in the lung when isotonic medium-treated PMN were given. However, PMN exposed to hypertonicity ex vivo failed to sequester in the lung. The viability of cells following in vitro treatment was confirmed by their capacity to exclude trypan blue. The integrity of the cell membrane (i.e., low permeability to monovalent ions) was also demonstrated by the fact that hypertonically treated cells maintained their transmembrane potential and were able to hyperpolarize after the addition of the potassium ionophore valinomycin (Fig. 6B). Considered together, these data support the concept that hypertonicity may exert its protection by a direct effect on PMNs, which precludes their sequestration in the lung.

Hypertonicity alters neutrophil surface expression of CD11b and L-selectin

The initial steps leading to PMN sequestration in the vasculature involve interactions between neutrophil surface receptors and counterligands on endothelial cells (25). To evaluate whether HTS might impair PMN adhesion molecule expression and thus contribute to the reduced PMN sequestration, blood was obtained from animals following each resuscitation protocol and was evaluated for adhesion molecule expression. Fig. 7 demonstrates CD11b expression in vivo in animals resuscitated with RL or HTS. RL animals showed a significant increase in CD11b at the end of resuscitation compared with studied CINC mRNA in whole lungs of animals resuscitated with RL or HTS. As shown in Fig. 5, the shock/LPS-induced increase in CINC mRNA did not differ between RL and HTS infusion. A representative blot is shown in Fig. 5A, and the mean of multiple studies normalized for G3PDH is shown in Fig. 5B. These data suggest that altered CINC does not account for the attenuation of PMN accumulation following HTS.

Effect of HTS resuscitation on lung chemokine expression

We recently showed that augmented pulmonary PMN sequestration in this model was mediated by increased expression of the C-X-C chemokine CINC in the lung (16). To determine whether HTS might exert its effect by suppressing CINC expression, we...
the preshock value. This was sustained over the ensuing 4 h until the time of sacrifice. By contrast, in the HTS-treated animals, CD11b levels did not increase compared with the preshock value over this time period. Fig. 8 shows levels of L-selectin in blood PMNs following the two resuscitation regimens. In animals resuscitated with RL, L-selectin levels remained stable over time, while in HTS-treated animals, there was a significant loss of surface L-selectin expression.

To determine whether the effect of HTS on adhesion molecules was a direct one, we studied their expression on human PMN in vitro following exposure to various levels of hyperosmolarity. While HTS had no effect on CD11b itself, a 4-h incubation in HTS prevented the increase in response to LPS, FMLP, and the phorbol ester PMA (Fig. 9A). CD11b expression on freshly isolated cells stimulated with FMLP or LPS did not differ from the expression

FIGURE 4. Lung histology after shock/resuscitation and LPS. A, Following RL resuscitation, histology of the lungs showed edema, intra-alveolar hemorrhage, and extensive neutrophil accumulation. A representative picture of three animals per group is shown. Hematoxylin-eosin stain; magnification, ×40. B, Following resuscitation with HTS, the histologic assessment of the lungs demonstrated marked improvement. A representative picture of three animals per group is shown. Hematoxylin-eosin stain; magnification, ×40.

FIGURE 5. Effect of the resuscitation regimen on lung CINC mRNA expression. At 4 h following LPS, lungs were recovered and processed for Northern blot analysis as described in Materials and Methods. A, Total lung RNA was obtained at sacrifice, probed for CINC mRNA, and detected by autoradiography. Equivalent RNA loading was confirmed by reprobing for G3PDH. A representative Northern blot of five animals per group is shown. B, Densitometric analysis of CINC mRNA normalized for G3PDH. The data are the mean ± SEM for five animals per group. *, p < 0.05 vs sham.
observed after a 4-h incubation in isotonic medium. The level of CD11b expression on PMA-stimulated freshly isolated cells was approximately 35% higher than that following a 4-h incubation in isotonic medium (Fig. 9A). Several studies have suggested that increased CD11b expression is not necessary for augmented function (26, 27). To discern whether exposure of neutrophils to hypertonicity alters CD11b function, adhesion to endothelial cells was evaluated (Fig. 9B). As expected, LPS-stimulated neutrophils exhibited increased adhesion to an endothelial cell monolayer pretreated with TNF-α. However, this rise was inhibited by pretreatment with hypertonic medium. Considered together, these findings suggest that hypertonicity is able to impair both LPS-induced up-regulation of CD11b surface expression and its activation. In addition, incubation in 350 mosmol in vitro caused a time-dependent reduction in L-selectin levels, an effect that was augmented by increasing the osmolarity to 500 mosmol (Fig. 9C). L-selectin levels on cells incubated for 4 h in isotonic medium did not differ

FIGURE 6. Effect of ex vivo hypertonicity on neutrophil sequestration in the lung. A, Sequestration of 51Cr-labeled neutrophils in animals following incubation in isotonic or hypertonic medium. Neutrophils were recovered from donor rats, labeled with 51Cr, and then incubated in either isotonic medium (iso PMN) or hypertonic medium (350 mosmol; ht PMN) for 4 h at 37°C. Cells were then resuspended in isotonic medium and injected into shock/LPS animals resuscitated with RL. At sacrifice, lungs were recovered and analyzed in the gamma spectrometer as indicated in Materials and Methods. The degree of neutrophil labeling was unaffected by incubation in either isotonic or hypertonic medium. The data are the mean ± SEM for three animals per group. *, p < 0.01 vs all other groups. Freshly isolated cells exhibited similar sequestration as those reported for cells incubated in isotonic medium for 4 h (fresh cells: sham, 2.5 ± 1.1%; shock, 17.4 ± 0.4%; n = 3). B, Membrane potential of neutrophils following incubation in either isotonic medium or hypertonic medium (500 mosmol) for 4 h at 37°C. Membrane potential was assessed using the anionic fluorescent probe diS-C3 as described in Materials and Methods. Where indicated, valinomycin (2 μM) and gramicidin (5 μM) were added to the cuvette. Two representative traces of three separate experiments are shown.

FIGURE 7. Effect of HTS resuscitation on CD11b expression on rat neutrophils. Blood was sequentially collected from an animal at the beginning of the study, 1 h after resuscitation immediately before LPS administration, and 4 h after LPS treatment. Whole blood was incubated with FITC-labeled anti-CD11b Ab, and after erythrocyte lysis was analyzed by flow cytometry. The data are the mean ± SEM for five animals per group. *, p < 0.05 vs sham and vs HTS at the indicated time points. For comparison, the starting control value for each animal was normalized to 100, and subsequent readings were compared to this.

FIGURE 8. Effect of HTS resuscitation on neutrophil L-selectin in vivo. Blood was sampled from animals at the beginning of the protocol before shock/resuscitation as well as 4 h after LPS administration. Neutrophils were isolated on a Ficoll gradient and incubated with FITC-labeled anti-L-selectin Ab before analysis by flow cytometry. The data represent the mean ± SEM of three animals at each time point. *, p < 0.05 vs sham and vs RL. For comparison, the starting control value for each animal was normalized to 100, and subsequent readings were compared to this.
from those in fresh cells and were significantly higher than those in cells incubated for 4 h in either 350 or 500 mosmol (Fig. 9C).

**Hypertonicity alters endothelial cell ICAM-1 expression**

Endothelial cell ICAM-1 is an important ligand for neutrophil CD11b and has been implicated in mediating neutrophil-endothelial interaction at sites of inflammation (25, 28). While HTS-induced alterations in PMNs were shown to be sufficient to account for the reduced leukosequestration, this did not rule out the possibility that endothelial cells might themselves be affected by this regimen. To determine the effect of HTS resuscitation on ICAM-1 expression, we examined ICAM-1 mRNA and protein expression in the lungs of animals following shock/LPS and either RL or HTS resuscitation. Four hours after LPS infusion, the RL-resuscitated animals showed a marked increase in ICAM-1 mRNA expression, as shown in a representative blot (Fig. 10A). Densitometric evaluation of several studies revealed an approximately 50% reduction in ICAM-1 mRNA expression in animals resuscitated with HTS (Fig. 10B). Similar reductions in whole lung ICAM-1 protein were observed in HTS-resuscitated animals compared with RL-treated animals (Fig. 10C).

**Discussion**

HTS resuscitation from hemorrhagic shock is known to be an effective means of restoring circulation in the traumatized patient. The present studies demonstrate that this resuscitation protocol may have additional effects that may serve to prevent the development of lung injury. Specifically, using a two-hit model of lung injury in the rat, hypertonic resuscitation from hemorrhagic shock was shown to lessen transpulmonary albumin leak, reduce pulmonary leukosequestration, and prevent histopathologic changes associated with lung injury compared with those in animals resuscitated with RL.

PMNs transiently incubated in hyperosmolar solution ex vivo failed to sequester in lungs of shock/LPS animals resuscitated...
regulation of CD11b might be invoked, given that CD11b is
(15). A comparable mechanism for impaired LPS-induced up-
ulated degranulation of secondary granules in neutrophils, as
range of 500 –700 mosmol significantly impaired FMLP-stim-
Hampton and colleagues reported that hyperosmolarity in the
were impaired by hypertonicity. Using an in vitro system,
level of CD11b expression and its activation in response to LPS
hibited its stimulated up-regulation in vivo. In vitro, both the
itant up-regulation of CD11b and activation, hypertonicity
While cellular activation might be expected to lead to concom-
shrinkage, a process that has been shown to stimulate various
phorylation in neutrophils and fibroblasts (18, 32). This may be
shrinkage has been reported to cause extensive tyrosine phos-
cades as well as shrinkage-related changes in the cytoskeleton
principle, both the induction of volume-sensitive signaling cas-
LPS-stimulated up-regulation and activation. The mechanisms
of hypertonicity on leukocyte sequestration in the lung vasculature
reduction in BAL neutrophils. Second, PMNs exposed to a hyper-
accounted for the effect. First, lung MPO levels, a measure of
increase in vitro using hyperosmolar solutions (350 mosmol) in-
for CD11/CD18 might be predicted. Based on this consider-
role in pulmonary leukosequestration is markedly dependent on the inflammatory
neutrophil sequestration focused on the effect of hypertonicity
the expression of CD11/CD18 and L-selectin. The rationale
requirement for a particular adhesion molecule in pulmonary
findings of CD18- and L-selectin-independent neutrophil se-
intratracheal LPS in these animals will result in comparable
expressions of ICAM-1 and CD18, detected by Western blotting. Total
inhibition of leukocyte accumulation was incomplete, suggest-
role for CD18-independent mechanisms. Recent studies
using adhesion molecule-deficient mice have provided signifi-
with RL. This finding suggests that hypertonicity-induced
changes in circulating PMNs may be sufficient to account for
the salutary effect of HTS resuscitation in this model. In this
regard, blockade of surface integrins or selectin molecules has
been shown to prevent neutrophil accumulation in various mod-
els of inflammation (25, 29–31). In the present studies, both in
vivo and in vitro data suggest that hypertonicity might exert its
effect on PMNs through altering adhesion molecule expression:
L-selectin via increased shedding and CD11b through impaired
LPS-stimulated up-regulation and activation. The mechanisms
underlying these alterations warrant further investigation.
In principle, both the induction of volume-sensitive signaling cas-
cades as well as shrinkage-related changes in the cytoskeleton
might contribute to the overall effect. For example, osmotic
shrinkage has been reported to cause extensive tyrosine phos-
phorylation in neutrophils and fibroblasts (18, 32). This may be
caused by clustering of surface receptors related to osmotic
shrinkage, a process that has been shown to stimulate various
receptors on the surface of HeLa in the absence of their ligands
(33). Relevant to the present studies, clustering of the Leu 13
receptor on lymphocytes has been shown to cause L-selectin
shedding through a tyrosine kinase-dependent mechanism (34).
While cellular activation might be expected to lead to concom-
titant up-regulation of CD11b and activation, hypertonicity
alone did not alter surface CD11b expression and actually in-
hibited its stimulated up-regulation in vivo. In vitro, both
the level of CD11b expression and its activation in response to LPS
were impaired by hypertonicity. Using an in vitro system,
Hampton and colleagues reported that hyperosmolarity in the
range of 500–700 mosmol significantly impaired FMLP-stim-
ulated degranulation of secondary granules in neutrophils, as
evidenced by reduced release of the granular enzyme lysozyme
(15). A comparable mechanism for impaired LPS-induced up-
regulation of CD11b might be invoked, given that CD11b is
also present to some extent in secondary granules as well as in
secretory vesicles in these cells (35). Recent studies testing the
effect of the actin-polymerizing agent jasplakinolide on neutro-
phil function revealed that this agent was able to prevent
FMLP-induced expression of CD11b in neutrophils (36), sug-
suggesting that the state of the actin cytoskeleton might influence
exocytic translocation of receptors to the cell surface. The
lar filamentous actin content is known to be increased fol-
lowing hypertonicity-induced cell shrinkage (37) and may
therefore have similarly contributed to the impaired LPS-stim-
ulated increase in CD11b both in vivo and in vitro.
In the present studies the investigations regarding altered
neutrophil sequestration focused on the effect of hypertonicity
on the expression of CD11/CD18 and L-selectin. The rationale
for choosing these particular adhesion molecules was based on
previous studies demonstrating their role in lung neutrophil ac-
cumulation and subsequent tissue damage in models of acute
lung injury. For example, inhibitory anti-CD18 mAb was shown to
markedly reduce neutrophil emigration into the alveolar space of rabbits following intratracheal instillation of LPS (31).
Similarly, anti-L-selectin Abs diminished lung neutrophil ac-
cumulation in a rodent ischemia/reperfusion model as well as in
an injury model induced by intrapulmonary instillation of IgG
complexes (38, 39). However, particularly in the first model,
inhibition of leukocyte accumulation was incomplete, suggest-
ing a role for CD18-independent mechanisms. Recent studies
using adhesion molecule-deficient mice have provided signifi-
cant support for this concept. In both L-selectin-deficient (40,
41) as well as CD18-deficient mice (41), neutrophil emigration
into the lung in response to intratracheal challenge with either
live Escherichia coli or Streptococcus pneumoniae was actually
increased compared with that in wild-type animals. Whether
intratracheal LPS in these animals will result in comparable
findings of CD18- and L-selectin-independent neutrophil se-
questration or findings similar to those reported in the afore-
mentioned Ab studies remain to be tested. However, since the
requirement for a particular adhesion molecule in pulmonary
leukosequestration is markedly dependent on the inflammatory
stimulus (31, 39), previous work would suggest that in the
shock/LPS model used in the present studies, a prominent role
for CD11/CD18 might be predicted. Based on this consider-
ation, it seems plausible that the ability of hypertonic medium
to inhibit up-regulation and activation of this adhesion complex
might contribute significantly to the inhibition of neutrophil
sequestration following hypertonic resuscitation. The approxi-
mately 30% reduction in L-selectin levels in hypertonic resus-
citated animals may be contributory, although alone it is un-
likely to have been responsible for the observed protection.
Other adhesion molecules may also be important and require
further study.
During the process of transmigration, neutrophils have been
shown to swell by up to 60% (42, 43). Preventing this volume
increase in vitro using hyperosmolar solutions (350 mosmol)
inhibited the chemotactic index by approximately 50% (43). In
the present studies we did not specifically evaluate the relative effects
of hypertonicity on leukocyte sequestration in the lung vasculature
vs transmigration of neutrophils into the alveolar space. However,
two lines of evidence suggest that reduced sequestration may have
accounted for the effect. First, lung MPO levels, a measure of
overall PMN sequestration, were lowered concomitant with the
reduction in BAL neutrophils. Second, PMNs exposed to a hyper-
tonic incubation ex vivo failed to accumulate in the lung tissue.
While these data together implicate reduced sequestration as a
mechanism for the effect of HTS, we cannot rule out the possibility

FIGURE 10. Effect of HTS resuscitation on ICAM-1 expression in rat
lungs. A, Total lung RNA was obtained at sacrifice, probed for ICAM-1
mRNA, and detected by autoradiography. Equivalent RNA loading was con-
firmed by reprobing for G3PDH. A representative Northern blot of three an-
imals per group is shown. B, Densitometric analysis of ICAM-1 mRNA nor-
malized for G3PDH. The data are the mean ± SEM for five animals per group.
*, p < 0.05 vs sham and vs HTS. C, ICAM-1 protein expression. After sac-
ifice, whole lungs were homogenized in 1% Triton, and equal amounts of
protein were separated on 15% SDS-PAGE, transferred to polyvinylidene dif-
luoride membranes, and probed for total ICAM-1. A representative Western
blot of five independent studies is shown.

Densitometric analysis of ICAM-1 mRNA normalized for G3PDH. A representative Northern blot of three animals per group is shown. B, Densitometric analysis of ICAM-1 mRNA normalized for G3PDH. The data are the mean ± SEM for five animals per group. *, p < 0.05 vs sham and vs HTS. C, ICAM-1 protein expression. After sacrifice, whole lungs were homogenized in 1% Triton, and equal amounts of protein were separated on 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed for total ICAM-1. A representative Western blot of five independent studies is shown.
that reduced transmigration may have additionally contributed to the diminution of BAL neutrophil counts in HTS-resuscitated animals.

Angle and colleagues recently reported that HTS resuscitation in a mouse hemorrhagic shock model caused a modest reduction in the lung injury score as well as an improvement in the histopathologic appearance of the lung (9). Neutrophil CD11b levels did not differ between control and HTS-resuscitated animals, while L-selectin, chemokine, and endothelial cell adhesion molecules were not examined. Importantly, the murine model differs significantly from the two-hit model in the rat presented herein, in that hemorrhage/resuscitation alone in the mouse suffices to induce inflammation in the lung concomitant with increased expression of proinflammatory and immunoregulatory cytokines (9). In man, hemorrhage/resuscitation alone rarely initiates such a profound response, suggesting that the two-hit model may be more representative of human disease (12, 13). Nevertheless, the murine model may prove valuable in dissecting out some of the mechanistic aspects of the salutary effects of hypertonic resuscitation.

In summary, the present studies demonstrate a beneficial immunomodulatory effect of hypertonic resuscitation on the development of lung injury. This effect appears to be due to an inability of circulating neutrophils to sequester at the site of injury. These findings suggest that the induction of a transient serum hyperosmolar-nomodulatory effect of hypertonic resuscitation on the development of lung injury by suppressing neutrophil activation after hemorrhagic shock. J. Trauma 44:59.

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In summary, the present studies demonstrate a beneficial immunomodulatory effect of hypertonic resuscitation on the development of lung injury. This effect appears to be due to an inability of circulating neutrophils to sequester at the site of injury. These findings suggest that the induction of a transient serum hyperosmolarity might minimize tissue injury in other inflammatory processes that are characterized by neutrophil infiltration and damage, such as those that occur following ischemia/reperfusion.

References