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Hemorrhagic Shock Primes for Increased Expression of Cytokine-Induced Neutrophil Chemoattractant in the Lung: Role in Pulmonary Inflammation Following Lipopolysaccharide

Jie Fan,* John C. Marshall,* Maria Jimenez,* Pang N. Shek, † John Zagorski, ‡ and Ori D. Rotstein2*

Recent studies have suggested that hemorrhagic shock followed by resuscitation renders patients more susceptible to lung injury by priming for an exaggerated response to a second stimulus, the so-called “two-hit” hypothesis. We investigated the role of C-X-C chemokines in mediating the augmented lung inflammation in response to LPS following resuscitated shock. In a rodent model, animals exposed to antecedent shock exhibited enhanced lung neutrophil sequestration and transpulmonary albumin flux in response to intratracheal LPS. This effect correlated with an exaggerated expression of cytokine-induced neutrophil chemoattractant (CINC) protein and mRNA, but not macrophage-inflammatory protein 2. Strategies designed to inhibit CINC, both anti-CINC Ab and supplementation with the antioxidant N-acetyl-cysteine, prevented the enhanced neutrophil sequestration, suggesting that CINC played a central role in the enhanced leukocyte accumulation following shock plus LPS treatment. Shock alone increased lung nuclear factor-κB expression and augmented the response to LPS. Prevention of this effect by N-acetyl-cysteine supplementation of the resuscitation fluid implicates a role for oxidant stress in the priming for lung inflammation following shock. Finally, alveolar macrophages recovered from shock-resuscitated animals released more CINC protein in vitro in response to LPS than macrophages from sham animals. Considered together, these findings show that augmented release of CINC, in part from primed alveolar macrophages, contributes significantly to the enhanced lung leukosequestration and transpulmonary albumin flux in response to LPS following resuscitated shock.


The adult respiratory distress syndrome (ARDS) is a common cause of morbidity following trauma (1, 2). Hemorrhagic shock is believed to contribute to the pathogenesis of ARDS by rendering the patient more susceptible to a second, seemingly trivial, inflammatory stimulus, the so-called “two-hit” model (3). Studies have suggested that ischemia-reperfusion can augment neutrophil-mediated lung injury by priming circulating neutrophils for increased superoxide production, with the result that cytotoxicity is enhanced once they are sequestered in the lung (4, 5). However, the mechanisms whereby pulmonary leukosequestration occurs in this model have not been elucidated.

The family of C-X-C chemokines are potent neutrophil chemoattractants that have been implicated in neutrophil influx to acute inflammatory sites (6). Patients with ARDS have elevated levels of IL-8 in their bronchoalveolar lavage fluid (BALF), and its correlation with the severity of the process suggests a role in the induction of lung inflammation (7–9). In rodent models of lung inflammation, two particular chemokines, cytokine-induced neutrophil chemoattractant (CINC) and macrophage-inflammatory protein-2 (MIP-2), have been shown to play a central role in neutrophil emigration into the lung (10–13). In the present studies, we hypothesized that increased lung C-X-C chemokine expression following hemorrhage/resuscitation might contribute to the increased susceptibility to lung injury following trauma. To test this possibility, we developed a “two-hit” lung injury model characterized by hemorrhagic shock with resuscitation, followed by intratracheal LPS administration. We show that the instillation of a low dose of intratracheal LPS following resuscitated shock causes a profound increase in lung neutrophil infiltration and transpulmonary albumin leak when compared with either hemorrhage/resuscitation alone or the administration of LPS alone. Concomitant with this effect, lung expression of CINC protein and mRNA in the LPS-treated shock animals was increased. The contribution of CINC to the development of lung injury in this model was demonstrated both by the ability of anti-CINC Abs as well as the antioxidant N-acetyl-cysteine (NAC) to prevent pulmonary leukosequestration. Alveolar macrophages recovered by BAL following hemorrhage/resuscitation period secreted more CINC protein in response to LPS than LPS-treated cells recovered from sham animals. Considered together, these findings suggest that alveolar macrophage priming for CINC expression following hemorrhagic shock plays an important role in the development of postresuscitation lung injury.

Materials and Methods

Animal model of hemorrhagic shock and lung injury

Male Sprague Dawley rats (300–350 g; Charles River, St. Constant, Quebec, Canada) were anesthetized with 80 mg/kg ketamine and 8 mg/kg...
sylamine administered i.p. 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 initiated 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 mean arterial pressure (MAP) was >45 mm Hg, and by infusion of 0.5 ml of Ringer’s lactate (RL) if the MAP was <35 mm Hg. Shed blood was collected into 0.1 ml citrate/ml blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood and RL in a volume equal to that of shed blood, over a period of 2 h. In some studies, animals received NAC (0.5 g/kg) via the artery before the infusion of RL. The catheter was then removed, the carotid artery was ligated, and the cervical incision was closed. Sham animals underwent the same surgical procedures, but hemorrhage was not induced. NAC delivery occurred in sham animals at an equivalent time to that received in shock animals.

At 1 hour after resuscitation, a tracheotomy was performed with a 14-gauge catheter, and either LPS (Escherichia coli O111B4, 30 μg/kg in 200 μl saline) or saline (SAL) alone was administered intratracheally, followed by 20 mechanically ventilated breaths using a rodent ventilator. The animals were therefore assigned to one of four groups: sham/SAL, shock/SAL, sham/LPS, and shock/LPS.

For in vivo anti-CINC Ab blockade experiment, rats were given an intratracheal instillation of 250 μg of rabbit anti serum against rat CINC or rabbit nonimmune IgG in 100 μl of SAL at 10 min before the intratracheal LPS instillation.

Animals were sacrificed at various time points by pentobarbitol overdose.

Bronchoalveolar lavage

For BAL, the lungs were lavaged via the intratracheal angiocath with cold PBS (8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4, with 0.1 mM EDTA). PBS was instilled in 10-ml aliquots and gently withdrawn with a 10-ml syringe to a total volume of 40 ml (14).

BAL fluid was centrifuged at 300 × g for 10 min to pellet cells. Supernatant was discarded, and the pellet cells were resuspended in a small volume of serum-free DMEM culture medium (Life Technologies, Burlington, Ontario, Canada). Total cell counts were determined on a grid hemocytometer. Differential cell counts were enumerated on cytoplasm-prepared slides that were stained with Wright-Giemsa stain. A total of 500 cells was counted in cross-section per sample, and the number of PMNs and alveolar macrophages was calculated as the total cell count times the percentage of the respective cell type in the BALF sample.

Assessment of transpulmonary albumin flux

Transpulmonary albumin flux was assessed by injecting 1 μCi of [35S]-labeled albumin in 0.2 ml of saline into the tail vein immediately following intratracheal LPS or SAL (14). At the end of the experimental protocol, 1 ml of blood was withdrawn by cardiac puncture for immediately following intratracheal LPS or SAL (14). At the end of the experiment, 1 ml of blood was withdrawn by cardiac puncture for counting cpm. Following exsanguination, lungs were perfused via a cannula in situ with 10 ml of PBS. The perfused PBS was withdrawn gently and aliquoted into 1 ml/tube for counting cpm. The transpulmonary albumin flux was normalized to blood cpm as follows:

**Northern blot analysis**

Total RNA from lungs was obtained using the guanidium-isothiocyanate method (15). Briefly, lungs were harvested from treated animals and immediately frozen in liquid nitrogen. Lungs were then thawed and homogenized in 10 ml of 4 M guanidine-isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, and 100 mM β-mercaptoethanol. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel, and transferred to nylon membrane.

Hybridization was conducted using a [32P]ATP end-labeled 30-base oligonucleotide probe for CINC with the sequence 5′-GGCCCAGCTCCTCTCCAACCTGCTTGAGTGCTT-3′, which is complementary to nucleotides 134 to 164 of CINC cDNA (see Ref. 12; sequence 5′-GCTCCGGGAATTTCCCTGGCCTGGA-3′).

A aliquot of 5 μg of nuclear protein was used together with the labeled double-stranded probe (~50,000 cpm) in the presence of 5 μg of nontarget, poly(dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM DTT) at 25°C for 20 min. Specific competition was performed by adding 100 ng of unlabeled double-stranded CINC oligonucleotide, while for nonspecific competition 2 μg of unlabeled double-stranded mutant CINC oligonucleotide (5′-CCTGGCTCCAATTTCCCTGCGCTGA-3′) that does not bind NF-kB was added. The mixture was separated by electrophoresis on a 5% polyacrylamide gel in 1× Tris glycine EDTA buffer (18). Gels were vacuum dried and subjected to autoradiography and PhosphorImager analysis.

**Western blot analysis**

Lung tissue homogenate samples or aliquots of macrophage supernatant were separated on an 15% SDS-PAGE under nonreducing condition (19). Equivalent loading of the gel was determined by quantification of protein, as well as by Coomassie blue staining of the gel. Separated proteins were electroblotted onto PVDF membrane and blocked for 1 h at room temperature with Tris-buffered SAL containing 1% BSA. The membranes were then incubated with a 1/1000 dilution of antiserum against rat CINC 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 exposed to the enhanced chemiluminescence detection system (Amer sham, Arlington Heights, IL), according to the manufacturer’s instructions.

**Ex vivo macrophage release of CINC**

To further evaluate the priming effect of hemorrhage-resuscitation on CINC generation, ex vivo experiments were performed using isolated alveolar macrophages. One hour after hemorrhage/resuscitation, BAL was performed on both shocked or sham animals. At this time point, there was no difference between groups with respect to the total cell count in the BAL fluid or the absolute number of alveolar macrophages recovered. A volume of 40 ml of BAL fluid was collected from each rat, and centrifuged at 300 × g for 10 min. The cell pellet was then suspended in NIM.2 neutrophil isolation medium (Cardinal Associates, Santa Fe, NM), and centrifuged at 750 × g, 20°C for 45 min for macrophage isolation (20). The isolated macrophages were washed in 5 ml of modified (calcium- and magnesium-free) HBSS and centrifuged again at 300 × g for 10 min. The pellet was resuspended in DMEM culture medium containing 10% FCS at a concentration of 1 × 106 cells/ml medium, and 1 ml was added to polypropylene tissue culture tubes. This technique generated a cell suspension with a viability of excess of 95%, as assessed by trypan blue exclusion, and a cell population of >95% macrophages, as assessed by Wright-Giemsa staining.

The macrophages obtained from hemorrhage-resuscitated or sham rats were then incubated for 1, 2, 4, and 6 h at 37°C in 5% CO2, either alone
Shock increases LPS-induced transpulmonary albumin flux

As shown in Figure 1A, neither shock alone nor a low dose of intratracheal LPS increased in transpulmonary albumin flux compared with sham animals given intratracheal SAL vehicle. By contrast, LPS administration following resuscitation from a period of hemorrhagic shock caused a threefold increase in the albumin flux. The priming effect of shock was also observed in the magnitude of LPS-induced lung neutrophil influx. While LPS alone caused a small increase in BALF neutrophil counts, animals subjected to shock before LPS exhibited a further threefold increase in neutrophil numbers (Fig. 1B). Shock alone caused neither an alveolar neutrophilia nor a rise in total cell count in the BALF compared with sham animals. Considered together, these data support a priming role for hemorrhagic shock in the LPS-induced pulmonary leukosequestration and increase in transcapillary albumin leak.

LPS-stimulated lung chemokine expression following hemorrhagic shock

CINC and MIP-2 both have been shown to participate in neutrophil infiltration into the lung following LPS administration (10, 11, 13). To determine whether enhanced expression of either or both of these chemokines might contribute to the priming effect of shock in this model, we examined their expression in animals treated with LPS with or without prior shock. Figure 2 illustrates representative Western blots of whole lung for each of these chemokine attractants. Shock alone had little effect on whole lung CINC protein, while LPS caused an increase by 4 h (Fig. 2A). However, antecedent shock followed by LPS caused a marked increase in CINC by 2 h, increasing further by 4 h. Figure 2B demonstrates levels of MIP-2. Both shock alone and LPS alone caused an increase in this protein by 4 h. In contrast to CINC expression, the levels of MIP-2 in shock/LPS animals were not consistently greater than sham/LPS at either 2 or 4 h.

To further examine the mechanism underlying the augmented CINC expression in shock/LPS animals, lung CINC mRNA levels at 4 h following LPS (or SAL vehicle) were evaluated (Fig. 3, A and B). Shock alone failed to induce CINC mRNA expression, while LPS caused a fourfold increase. However, the effect of LPS was markedly enhanced by prior shock/resuscitation (~eightfold compared with sham/SAL, \( p < 0.01 \) vs sham/LPS). The priming was also observed in time course studies (Fig. 3, C and D). Sham/LPS caused a slight increase by 2 h with clear up-regulation by 4 h. By contrast, CINC mRNA expression was observed in shock/LPS animals as early as 1 h and remained elevated above sham/LPS for up to 4 h.

The promoter region of the CINC gene contains an NF-\( \kappa \)B consensus binding sequence that is believed to contribute to the regulation of gene transcription (12, 21–23). Previous studies have reported the ability of shock to induce NF-\( \kappa \)B translocation in the lung mononuclear cells using the \( \kappa \)B DNA sequence from the Ig gene (24, 25). In the present studies, we evaluated the effect of shock on NF-\( \kappa \)B translocation using the \( \kappa \)B DNA sequence from the CINC gene. Gel-shift assays on whole lungs were performed to

Statistics

The data are presented as mean ± SE of \( n \) determinations, as indicated in the figure legends. Data were analyzed by one-way analysis of variance; post hoc testing was performed using the Bonferroni modification of the \( t \) test. When individual studies are demonstrated, these are representative of at least three independent studies.

Results

Shock increases LPS-induced transpulmonary albumin flux

or in the presence of 0.5 \( \mu \)g/ml of LPS. At the end of the incubation period, cells were sedimented by centrifugation at 300 \( \times \) g for 10 min. Supernatants were aspirated and frozen at −70°C for later measurement of CINC by Western blotting.

FIGURE 1. A, Alterations in transpulmonary albumin flux 6 h after treatment with intratracheal LPS (30 \( \mu \)g/kg) or SAL. Transpulmonary albumin flux was assessed as described in Materials and Methods. The data represent the mean ± SEM of studies performed in four rats in each group. **\( p < 0.01 \) vs other groups. B, BAL neutrophil counts flux in sham or shock rats 4 h after treatment with intratracheal LPS (30 \( \mu \)g/kg) or SAL. The PMN counts increased 10-fold in sham/LPS animals (*\( p < 0.05 \)) and 38.3-fold in shock/LPS animals (**\( p < 0.01 \)) compared with sham/SAL group. Shock/LPS was significantly greater than sham/LPS (*\( p < 0.01 \)). The data represent the mean ± SEM of studies performed in four rats in each group.

FIGURE 2. A, Effect of shock on LPS-induced CINC expression in lung tissue. Western blot with anti-CINC Ab was performed on lung tissue sampled from shocked or sham rats at 2 and 4 h after LPS or SAL intratracheally. A representative of three independent experiments is shown. B, Effect of shock on LPS-induced MIP-2 expression in lung tissue. Western blot for MIP-2 was conducted on the same lung tissue samples as A described above. A representative of three independent experiments is shown.

LPS-stimulated lung chemokine expression following hemorrhagic shock

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discern whether increased nuclear translocation of NF-κB might contribute to the priming observed in animals exposed to prior shock. As demonstrated in Figure 4, sham/LPS animals exhibit a slight increase in NF-κB translocation at 1 h, which reaches a maximum at 2 h and fades over the ensuing 4 h. In shocked animals, there is evidence for NF-κB translocation before LPS administration (t = 0 h). Following LPS treatment, the increase is clearly present at 1 h, reaching a maximum at 2 h and dissipating over the next 4 h.

Anti-CINC Ab or NAC treatment prevents shock-induced priming

The data presented above show that the priming effect of hemorrhagic shock is associated with increased NF-κB translocation and an increased rate and magnitude of CINC expression in the lung. Two approaches were used to determine a causative role for CINC in the enhanced PMN influx in animals subjected to shock/LPS. First, the effect of intratracheal anti-CINC Ab was examined. As shown in Figure 5, anti-CINC Ab caused a ∼66% reduction in BALF neutrophil counts in sham/LPS animals compared with nonspecific IgG. In shock/LPS animals, anti-CINC Ab totally abrogated the shock-induced rise in alveolar neutrophil sequestration and further reduced neutrophil levels to those observed in sham/LPS animals treated with anti-CINC. These findings suggest that the augmented influx of neutrophils in the shock/LPS animals requires the enhanced expression of CINC.
Role of oxidant-induced NF-κB translocation in shock priming

Previous studies have implicated a role for oxidants in the induction of increased NF-κB translocation following shock (24). To determine whether oxidants might contribute to the shock-induced priming for lung CINC expression, the effect of NAC on NF-κB translocation was studied. Figure 8A is a representative study illustrating NF-κB translocation at 2 h following intratracheal SAL or LPS in sham or shock animals. Both sham/LPS (lane 3) and shock/SAL (lane 5) induced increases in NF-κB translocation, while antecedent shock primed the lung for a marked enhancement of NF-κB translocation in response to LPS (lane 7). Inclusion of NAC in the resuscitation fluid completely prevented the shock-induced priming for increased NF-κB translocation in shock/LPS animals (lane 8). Treatment of sham animals with NAC at 1 h before intratracheal LPS instillation caused a small reduction in NF-κB translocation, while NAC caused a significant reduction in NF-κB translocation following shock alone. Densitometric data showing the average of four animals per group are demonstrated in Figure 8B. In conjunction with the data regarding the effect of NAC on CINC expression, these findings suggest that shock induces an oxidant stress that primes for enhanced NF-κB translocation and CINC expression following exposure to LPS.

Alveolar macrophages are primed for CINC release

CINC is produced by a variety of cells, including neutrophils, epithelial cells, and macrophages (26). To determine whether alveolar macrophages contributed to the enhanced whole lung CINC.
expression, BAL cells were recovered at the end of resuscitation and enriched for macrophages. These cells were then incubated ex vivo in the presence or absence of LPS to evaluate CINC release. As shown in Figure 9, CINC is released from the macrophages of shock animals as early as 1 h following treatment with LPS, reaching a peak at 2 h and then decreasing slightly at 4 h. By contrast, LPS-treated macrophages from sham animals generated a small quantity of CINC at 4 h. Similarly, supernatant derived from untreated macrophages, either from sham or shock animals, contained small and inconsistent levels of CINC. These data demonstrate that alveolar macrophages may be one of the sources of increased CINC release in animals following shock/resuscitation and subsequent exposure to LPS.

Discussion

Recent studies have supported a “two-hit” hypothesis in the pathogenesis of lung injury in trauma patients, namely an initial stimulus may prime for subsequent organ damage in response to a second, often minor, insult (3). One possible mechanism underlying this phenomenon may be that circulating neutrophils are primed for increased lung sequestration and cytotoxic activity (5). Furthermore, circulating neutrophils derived from rodents following ischemia/reperfusion of the gut exhibited augmented superoxide and CD11b expression in response to a second stimulus. Interestingly, when neutrophils derived from these animals were transfused into normal animals, they failed to sequester in the lungs, suggesting that perturbations at the organ level may also contribute to neutrophil accumulation and lung injury (27). The present study is the first to describe hemorrhagic shock-induced priming for lung chemokine generation as a mechanism contributing to neutrophil sequestration and consequent increased albumin flux in response to a second stimulus.

Previous studies have shown that both CINC and MIP-2 are increased in the lung in response to LPS (10–13). These reports further suggest that both chemokines might contribute to neutrophil sequestration, as evidenced by the fact that specific neutralizing Abs directed against either of these chemokines caused partial inhibition of neutrophil influx. Shanley and colleagues reported similar findings in a model of immune complex-induced lung injury (28). Consistent with these reports, the present studies demonstrate that LPS, without prior shock, increases both CINC and MIP-2 protein in the lung. The present data suggest, however, that the augmented neutrophil influx in response to LPS in animals subjected to shock/resuscitation is primarily due to a differential increase in CINC expression. Several lines of evidence support this conclusion. First, CINC levels were increased in shock/LPS animals compared with LPS alone, while MIP-2 levels in response to LPS did not differ between shock and sham. Second, anti-CINC Ab completely reversed the shock-induced augmentation in BALF neutrophils in response to LPS. Finally, the ability of NAC treatment to reduce CINC levels in shock/LPS animals to those detected in sham/LPS animals correlated with its ability to prevent the increased neutrophil influx observed in the shock/LPS animals. Considered together, these findings provide strong evidence that antecedent shock selectively primed for increased CINC expression, and this accounted for the augmented neutrophil sequestration and consequent lung injury in shock/LPS animals.

The augmented CINC production in the lung appears to be related to increased levels of CINC gene expression. Previous studies have suggested that activation of xanthine oxidase during hemorrhage/resuscitation may lead to inflammatory gene activation through oxidant-induced NF-κB translocation (24, 29). Our studies...
show that shock alone may cause a small increase in NF-κB translocation by a similar mechanism, since the effect is partially prevented with the antioxidant NAC. Furthermore, we believe that the increased NF-κB in animals at the end of shock/resuscitation is indicative of a primed macrophage in the lung. The primed state is evidenced by a more rapid and heightened translocation of NF-κB and expression of CINC mRNA in response to LPS. In this regard, Mendez et al. (30) showed that macrophages exposed to oxidants in vitro exhibited increased NF-κB translocation and levels of TNF mRNA following LPS. Two lines of evidence support this experiment in resident macrophages rather than those recruited into the lung. First, macrophage numbers in the BAL do not differ between sham and shock animals at the end of resuscitation and before LPS. Second, when these macrophages are recovered and stimulated in vitro, the shock macrophages generate more CINC protein. Thus, these primed resident macrophages are sufficient to explain increased CINC, and thus enhanced neutrophil sequestration. Different sensitivities to the effects of oxidants may be one possible explanation for the differential priming for CINC vs MIP-2 expression following shock. In addition to xanthine oxidase, release of endogenous LPS may also have contributed to NF-κB activation through oxidant generation. Circulating LPS is known to be increased transiently following hemorrhage/resuscitation (31), and studies have shown its ability to stimulate NF-κB expression both in vivo and in vitro in an oxidant-dependent manner (12, 32). This possibility is consistent with studies by Smith and colleagues demonstrating the ability of systemic endotoxin administration to prime alveolar macrophages for increased production of inflammatory mediators in response to a second dose of endotoxin (33). Further investigation is required to define the precise nature of the oxidant stress, its source, and its effect on lung chemokine expression.

The present studies provide data that show similarities to those in a recent report evaluating the effect of NAC on lung injury following the i.p. administration of LPS (12). The finding that NAC caused a small reduction in NF-κB binding in sham/LPS animals was similar to that described by Blackwell and colleagues (12), consistent with in vitro data demonstrating an effect of redox manipulation on LPS-induced NF-κB translocation (32, 34). In the study by Blackwell and colleagues, NAC caused a 48% reduction in CINC mRNA expression and complete prevention of neutrophil influx (12), while our data show little effect on CINC mRNA or neutrophil number in the BALF in sham/LPS animals. The disparity may in part be related to differences in experimental models. For example, whereas our neutrophil determinations were performed at 4 h after a single dose of NAC, studies by Blackwell et al. (12) were performed at 24 h following multiple NAC doses. Furthermore, the route of administration of LPS may influence the local cytokine milieu in the lung, and possibly the response to antioxidant therapy (35).

Recent clinical reports have demonstrated a beneficial effect of antioxidant therapy in patients with established ARDS (6, 36, 37). The experiments presented in this study suggest the intriguing possibility that the use of antioxidants in the resuscitation fluid of hypotensive patients may lessen their susceptibility to the subsequent development of ARDS in response to a second stimulus. This approach may be applicable not only to trauma victims, but also to patients undergoing major surgical procedures.

References