Resuscitation with lactated Ringer solution limits the expression of molecular events associated with lung injury after hemorrhage

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Resuscitation with lactated Ringer solution limits the expression of molecular events associated with lung injury after hemorrhage. J Appl Physiol 98: 550–556, 2005. First published October 15, 2004; doi:10.1152/japplphysiol.00858.2004.—The aim of this study was to determine whether hemorrhage altered the caspase-3 activity and the ATP levels in rat lung and ileum tissues and determine whether resuscitation with lactated Ringer solution (LR) or whole blood (WB) reversed these changes. Male Sprague-Dawley rats were briefly anesthetized with isoflurane, and their mean arterial blood pressure was reduced from 110 to 40 mmHg by bleeding. The bled rat was then resuscitated with LR or autologous WB to bring mean arterial blood pressure back to 80 mmHg. Lung and ileum tissues were removed at the end of hemorrhage or at the end of the resuscitation period for specified bioassays. Hemorrhage increased cellular caspase-3 activity in the lung and the ileum. After the hemorrhaged rats received LR or WB, caspase-3 activity returned to the basal level in the lung and ileum, respectively. Likewise, hemorrhage decreased cellular ATP levels in lung and ileum. After LR or WB resuscitation, the cellular ATP level returned to the basal level only in the lung resuscitated with LR. The increased caspase-3 activity was associated with the increased expression of caspase-3 mRNA, which also returned to normal levels after either resuscitation. Similarly, hemorrhage increased the expression of inducible nitric oxide synthase and Kruppel-like factor 6 and decreased expression of Kruppel-like factor 4. Subsequent LR resuscitation normalized the expression of these genes in the lung tissue. Our results demonstrate that resuscitation with LR can reverse the expression of genes and their products that are thought to contribute to hemorrhage-induced lung injury.

Kruppel-like factor 6 and 4; inducible nitric oxide synthase; whole blood

Hemorrhage has been shown to lead to systemic inflammatory response syndrome and multiple organ dysfunction and failure (6). A variety of molecules, including stress-related proteins, has been known to be involved in this response (24, 25). Various approaches to reduce the hemorrhage-induced injury, including stimulation of cytoprotectant proteins or inhibition of the expression of the injury-causing proteins or pathways, have been attempted. For example, in rodent models, increases in inducible heat shock protein 70 (HSP70i) by heat stress or chemical stimulation limit tissue injury caused by ischemia-reperfusion (14, 43), hemorrhage (24, 25, 30), or hemorrhage followed by resuscitation (40). In transgenic mice expressing HSP70i, protection against myocardial dysfunction after brief ischemia has been reported (45). Likewise, inhibition of nitric oxide production results in significant reduction of local tissue damage, polymorphonuclear neutrophils infiltration, and leukotriene B4 generation caused by ischemia-reperfusion (8). Mice deficient in inducible nitric oxide synthase (iNOS) also demonstrate limited hemorrhage/resuscitation-induced injury (19, 32). Therefore, interventions that induce HSP70i and/or inhibit iNOS might be useful in the reduction of hemorrhage/resuscitation-induced injury.

In vitro studies have shown similar results. Low oxygen supply simulating ischemia or hemorrhage affects the expression of iNOS, which then influences the expression of other proteins that alter cell viability. It has been shown that hypoxia results in alteration of iNOS, Bcl-2, and p53 mRNA expression in cultured human intestinal epithelial T84 cells and Jurkat T cells (26, 50) and that these alterations can be modulated by treatment with a NOS inhibitor (26). It has also been found that hypoxia increases the activity of caspase-3, an aspartate-specific cysteinyI protease involved in apoptosis, an activity that is blocked by NOS inhibitors (26).

A full time-course study of the effect of hemorrhage on a series of stress-related proteins, such as c-jun, Kruppel-like factor (KLF) 6, iNOS, HSP70i, and hypoxia-inducible factor-1α has been reported in a hemorrhage mouse model. It indicated the order of protein appearance to be c-jun, KLF6, iNOS, HSP70i, and hypoxia-inducible factor-1α (24). KLF4 (a repressor to iNOS; Ref. 50) was not detected. In addition to changes in this series of stress-related proteins listed above, hemorrhage also increases cellular caspase-3 activity (25) and reduces cellular ATP levels (9, 38, 47).

Although various fluids have been considered and been used to resuscitate individuals suffering loss of blood, there is no consensus on which is the best in terms of limiting organ injury. It is obvious that the resuscitation fluids that correct cellular/molecular pathological process that started during the hemorrhage period would be preferred to be used in a clinical setting. In this study, we demonstrate that lactated Ringer solution (LR), a fluid recommended by Acute Trauma Life Support Standard Care (22), can successfully reverse molecular changes initiated by hemorrhage in the lung but not in the ileum of rats. Resuscitation with whole blood (WB) (3, 13, 15, 16, 23, 37, 41, 44, 46) was also studied as a control. Plasma and normal saline were not chosen because plasma resuscitation alone is known to increase caspase-3 activity (7), and normal saline resuscitation caused further kidney damage (37) and lung injury (13, 46).
METHODS

**Experimental protocol.** Male Sprague-Dawley rats weighing between 350 and 400 g were used for this study. The hemorrhage-resuscitation was studied using the modified Wiggers model (51). Rats were briefly anesthetized with isoflurane (5% induction, 1–2% maintenance, Minrad, Buffalo, NY) to cannulate sterile, heparin-flushed Renepulse catheters (Braintree Scientific, Braintree, PA) into femoral artery and femoral vein. The catheters were then connected to the corresponding fluid reservoir and blood pressure monitor (BPA-400, Micro-Med, Louisville, KY) to record the mean arterial blood pressure.

Fig. 1. Hemodynamic changes before, during, and after hemorrhagic hypotension and resuscitation. A representative tracing from 1 rat is presented here. The detailed procedure is described in MATERIALS. SBV, shed blood volume; LR, lactated Ringer solution; MAP, mean arterial blood pressure.

Fig. 2. Resuscitation with LR or whole blood (WB) reduces the hemorrhage-induced increase in caspase-3 activity in the lung (A) and ileum (B), respectively. Caspase-3 activity in lysates of lung and ileum of sham-operated (sham; n = 6), hemorrhaged (HE; n = 9), and hemorrhaged plus LR- (HE + LR; n = 4) and WB-resuscitated (HE + WB; n = 4) rats was measured. A: significant difference determined by 1-way ANOVA and Bonferroni's inequality: *P < 0.05 vs. sham-operated group and HE + LR group; **P < 0.05 vs. sham group, HE group, and HE + WB. B: significant difference determined by 1-way ANOVA and Bonferroni's inequality: *P < 0.05 vs. sham-operated group and HE + WR group; **P < 0.05 vs. sham group, HE group, and HE + LR.

Fig. 3. LR resuscitation restores the hemorrhage-induced ATP loss in the lung. ATP levels in lysates of lung (A) and ileum (B) of sham (n = 6), HE (n = 7), and HE + LR (n = 4) rats was measured. A: significant difference determined by 1-way ANOVA and Bonferroni's inequality: *P < 0.05 vs. sham. B: significant difference determined by 1-way ANOVA and Bonferroni’s inequality: *P < 0.05 vs. sham.
sure (MAP). The rate of hemorrhage was controlled by a small pump (model 720, Instech, Plymouth Meeting, PA) for bleeding and resuscitation. MAP, heart rate, shed blood volume, and infusion fluid volume were recorded every 5 s using a data-acquisition program (LabView, National Instruments, Austin, TX). Figure 1 shows that the rats were stabilized for 20 min with their MAP maintained at ~110 mmHg. Then, MAP was reduced from 110 to 40 mmHg within 15 min by withdrawing blood to allow hemorrhage to occur and retained at this level for 30 min. The shed blood volume was 12.38 ± 0.6 ml (n = 10). The hemorrhaged rat was then resuscitated with LR to bring the MAP back to 80 mmHg within 15 min and stayed at that MAP for an additional 45 min. The volume of LR was 17.5 ± 4.4 ml (n = 6). In a parallel experiment, a group of hemorrhaged rats were resuscitated with shed autologous WB. Lung and ileum were removed at the end of hemorrhage or at the end of the resuscitation period and frozen at −70°C until use for specified bioassays.

Tissues were minced and sonicated for 15 s and then centrifuged at 10,000 g for 10 min. The supernatant was added with 0.225 ml of isoprolyl alcohol and mixed gently by inversion. The mixture was centrifugated at 12,000 g at 4°C for 15 min for RNA precipitation that was washed with 70% ethanol. The RNA pellet was air dried and then dissolved in RNase-free water.

RT-PCR. Using modified QIAGEN OneStep RT-PCR kit for half-quantitative RT-PCR (Qiagen, Valencia, CA), RT-PCR of caspase-3, KLF4, KLF6, Bcl-2, p53, iNOS, p38-MAPK, and β-actin was conducted. These RT-PCR products of mRNA of interested genes were normalized with β-actin because β-actin gene was not altered by hemorrhage or hemorrhage plus resuscitation (24).

Measurements of caspase-3 activity. Caspase-3 activity was determined using the CASPASE-3 cellular activity assay kit PLUS (Biomol, Plymouth Meeting, PA). Change in absorbance was measured at 405 nm with a SpectraMax 250 spectrophotometric plate reader and SOFTmax Pro 3.1.1 software (Molecular Devices, San Diego, CA). Data were normalized to total protein, and caspase-3 activity was expressed (in pmol pNA min⁻¹ μg protein⁻¹).

Measurements of cellular ATP level. Cellular ATP levels were determined using the ATP bioluminescence assay kit HS II (Roche, Mannheim, Germany). Luminescence was measured with TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Data were normalized

![Fig. 4](http://jap.physiology.org/).

**Fig. 4.** Resuscitation with either LR or WB reverses the hemorrhage-induced changes in caspase-3 and p38 mitogen-activating protein kinase (MAPK) gene expressions in the lung (A and B) and ileum (C and D). Using RT-PCR, mRNA of caspase-3, p38 MAPK, p53, Bcl-2, and β-actin genes was assessed in lung and ileum of sham (n = 6), HE (n = 9), and HE + LR (n = 4) or HE + WB (n = 4) rats. Three independent experiments were conducted. Their specific bands of mRNA were quantitated densitometrically and normalized with β-actin mRNA. Significant difference determined by 1-way ANOVA and Bonferroni’s inequality: *P < 0.05 vs. sham; **P ≤ 0.05 vs. sham and HE; ***P ≤ 0.05 vs. sham, HE, and HE + LR.
RESULTS

Viability. Hemorrhaged rats did not survive if bleeding lasted longer than 1 h unless they received resuscitation fluids to restore their MAP to the level of 80 mmHg. Therefore, we reduced the duration of the hemorrhage to 45 min to keep them alive to study changes in specific biomarker levels and related gene expression. Lung and ileum were chosen for studies here because lung is involved in delayed hemorrhage-induced injury that may lead to multiple organ dysfunction and failure, and ileum responds to hemorrhage promptly and is capable of recovery within hours (24, 25).

LR and WB reduce hemorrhage-induced increases in caspase-3 activity in the lung and ileum, respectively. Because hypoxia has been shown to alter cellular caspase-3 activity in human cultured cells (26), and it is considered to be a reliable biomarker of apoptosis, we decided to determine whether hemorrhage alters caspase-3 activity in rats subjected to hemorrhage. Hemorrhage increased cellular caspase-3 activity in lung and ileum tissues by 454 ± 35% (n = 6) and 322 ± 10% (n = 9), respectively. When the hemorrhaged rat was resuscitated with either LR or WB, caspase-3 activity returned to the basal level in the lung (Fig. 2A) and in the ileum (Fig. 2B), respectively.

LR and WB inhibit hemorrhage-induced increase in caspase-3 mRNA in lung and ileum, respectively. Increased caspase-3 activity in rats subjected to hemorrhage was associated with increased caspase-3 mRNA, which was blocked by the subsequent resuscitation. Resuscitation with LR (Fig. 4, A and B) or WB (Fig. 4, C and D) inhibited hemorrhage-induced increases in caspase-3 mRNA in lung and ileum, respectively.

LR and WB inhibit hemorrhage-induced increase in p38-mitogen-activated protein kinase mRNA in lung. p38 Mitogen-activated protein kinase (MAPK) is increased in tissues of animals subjected to hemorrhage (5, 21), which leads to increased TNF-α levels. Indeed, hemorrhage increased the expression of p38 MAPK mRNA in the lung and the ileum of rats subjected to hemorrhage. Subsequent LR resuscitation fully inhibited this increase in lung and ileum, but WB resuscitation inhibited the increase only in lung (Fig. 4).

Fig. 5. Resuscitation with LR or WB reverses the hemorrhage-induced changes in inducible nitric oxide synthase (iNOS) and its gene expressions in the lung (A and B) and ileum (C and D). Using RT-PCR, mRNA of iNOS, Kreppel-like factor (KLF) 4, KLF6, and β-actin genes was assessed in lung and ileum of sham (n = 6), HE (n = 9), and HE + LR (n = 4) or HE + WB (n = 4) rats. Three independent experiments were conducted. Their specific bands of mRNA were quantitated densitometrically and normalized with β-actin mRNA. Significant difference determined by 1-way ANOVA and Bonferroni’s inequality: *P < 0.05 vs. sham; **P ≤ 0.05 vs. sham and HE; ***P ≤ 0.05 vs. sham, HE, and HE + LR.
WB but not LR inhibits hemorrhage-induced increases in mRNA of p53 and Bcl-2 in lung and in Bcl-2 mRNA in ileum. Hemorrhage also induced increases in mRNA of p53 and Bcl-2 in lung and Bcl-2 in ileum. In contrast to p38 MAPK, LR resuscitation failed to block the increases of the expression of these genes, but WB resuscitation significantly inhibited their mRNA overexpressions (Fig. 4).

LR and WB inhibit hemorrhage-induced increases in iNOS mRNA in lung and ileum, respectively. It has been established that hemorrhage upregulates significantly the expression of iNOS in mouse lung (19, 24) and other organs (24). Because our laboratory has shown that inhibition of iNOS results in decreases in caspase-3 activity in human intestinal epithelial cells and Jurkat T cells (26), we asked whether resuscitation with LR or WB can reduce the hemorrhage-induced altered expression of iNOS. Indeed, hemorrhage increased iNOS mRNA in both lung and ileum, and subsequent resuscitation with LR or WB statistically significantly inhibited the increase in lung (Fig. 5, A and B) and ileum (Fig. 5, C and D), respectively. This observation suggests that the effect of resuscitation on the caspase-3 activity may be associated with the effect of the expression of iNOS.

It has been known that KLF4 and KLF6 are transcriptional factors that control the expression of the iNOS gene. Warke et al. (50) reported that KLF6 upregulated iNOS, whereas KLF4 downregulated it. In the rat lung, hemorrhage increased KLF6 mRNA and decreased KLF4 mRNA. The subsequent LR resuscitation decreased KLF6 mRNA to the basal level and increased KLF4 mRNA to its original basal level; WB resuscitation returned KLF4 mRNA in lung but showed no effect on the hemorrhage-induced increase in KLF6 mRNA (Fig. 5, A and B). It appears, therefore, that the effect of LR and WB resuscitation on iNOS is mediated by preceding effects on transcription factors known to control the expression of iNOS. KLF4 is probably more influential than KLF6 in this regard.

Hemorrhage decreased KLF4 mRNA and increased KLF6 mRNA in ileum, and resuscitation with LR or WB reduced KLF6 mRNA expression. Interestingly, LR and WB had only a slight effect on the expression of KLF4 mRNA (Fig. 5, C and D). This result suggests that KLF6 is more important than KLF4 in the regulation of the expression of iNOS in tissues subjected to hemorrhage and resuscitation.

**DISCUSSION**

Hemorrhagic shock is known to cause delayed detrimental effects such as multiple organ dysfunction (6, 24). The consensus is that a successful resuscitation fluid should be able to reverse cellular/molecular alteration caused by hemorrhage. For this reason, we sought to investigate the ability of LR (an Acute Trauma Life Support Standard Care recommended resuscitation fluid) on hemorrhage-induced changes in the expression of molecules directly related to organ injury, whereas WB was studied as a control.

In this study, hemorrhage was found to cause a significant increase in cellular caspase-3 activity. Previously, our laboratory had observed, in a mouse hemorrhage model, an increased caspase-3 activity in the lung after hemorrhage for up to 48 h, whereas in the small intestine the increase reached a maximum 6 h after hemorrhage and returned to the basal level within 12 h (24, 25). Caspase-3 is an aspartate-specific cysteinyl protease that plays a key role in cell apoptosis (20). It has been shown that caspase-3 activity is increased by hypoxia (17, 26) during hypoxia ischemia (12, 36, 49) and hemorrhage (11, 28, 29, 31, 33, 53) and in other pathological conditions (1, 34, 35, 39, 42, 48). Its inhibition by specific caspase-3 inhibitors (53), treadmill exercise (28), or hypertonic saline resuscitation (33) has been shown to reduce tissue apoptosis and brain damage in hemorrhaged animals. We report here that resuscitation of rats subjected to hemorrhage with LR or WB inhibited caspase-3 activity in lung and ileum, respectively. The reasons for this discrepancy between LR and WB are not known. It is possible that a rapid activation of alternate caspases (52) or increased sensitivity of the changes in the microenvironment of ileum (4) may account for the difference.

In our rat model, hemorrhage significantly reduced ATP levels in both lung and ileum. Similar results have been reported by other laboratories (9, 10, 38, 47). The subsequent LR resuscitation restored the ATP level in lung, which is consistent with the finding observed in the rat liver resuscitated with Ringer solution (38). The underlying mechanism is not clear. However, our preliminary data from an in vitro experiment (J. G. Kiang, unpublished data) indicate that DEVD inhibitor (a specific caspase-3 inhibitor) can reduce the ATP level, implying that ATP levels somehow are associated with the level of caspase-3 activity. WB resuscitation failed to correct the hemorrhage-induced ATP reduction in both the lung and the ileum, however.

**Fig. 6. Schematic representation of model in rat lung for iNOS-mediated pathways after hemorrhage and points where resuscitation with LR might block changes. Hemorrhage increases KLF6 and decreases KLF4, resulting in an increased expression of iNOS. This increase leads to increases in caspase-3 activity and ATP loss. Therefore, apoptosis occurs. Resuscitation with LR inhibits KLF6 and increases KLF4. Subsequently, iNOS expression is reduced followed by reduction in caspase-3 activity and ATP loss, and apoptosis is reduced. ↑, Increase; ↓, decrease.**
Our laboratory’s previous in vitro data (26) and the present in vivo data strongly indicate that increased caspase-3 activity correlates well with the upregulated iNOS expression. Similar results were found with caspase-3 and p38 MAPK in the lung and ileum. Our present observations in lung on hemorrhage-induced alteration are consistent with the report from Warke et al. (50) that showed that, in cultured cells, KLF6 stimulated iNOS overexpression, whereas KLF4 inhibited it. However, in ileum, KLF6 but not KLF4 seems to play a key role in regulating iNOS expression (24). Our findings, taken together with previously published results (26), suggest that iNOS plays an important role in controlling the caspase-3 activity and probably also the ATP levels in lung.

LR is effective in this regard to reduce caspase-3 activity (Fig. 2) and restore the ATP level (Fig. 3) in rat lung and in rat liver (38), but LR did not inhibit hemorrhage-induced increased expression of p53 and Bcl-2 in the lung, suggesting that LR is specific to iNOS-mediated pathways. Although LR has been reported to cause significant neutrophil activation (3), pulmonary apoptosis (2, 27), and increased expression of ICAM-1 (2), TNF-α, IL-1α, IL-5, IL-6, IL-7, IL-10, and IL-16 (2, 18), in the present study we found that LR increases KLF4, inhibits iNOS, KLF6, and caspase-3 activity, and restores ATP levels. Our findings reinforce the view that iNOS plays a very important role in organ injury caused by hemorrhagic shock. Figure 6 is a schematic representation of the findings in rat lung of this study. Hemorrhage increases KLF6 and decreases KLF4, resulting in an increased expression of iNOS. This increase leads to increases in caspase-3 activity and ATP loss. Resuscitation with LR inhibits KLF6 and increases KLF4. Subsequently, iNOS expression is reduced followed by reduction of caspase-3 activity, ATP loss, and apoptosis.

Resuscitation with WB also showed inhibition on molecular events associated with hemorrhage-induced injury but failed to restore ATP levels. WB has also been shown no improvement in metabolic acidosis, hyperventilation, and a significant interstitial PO2 decrease in hemorrhaged hamsters (23). In addition, WB resuscitation reduced mRNA of p53 and Bcl-2, whereas LR resuscitation did not. Therefore, LR should be a considerably good resuscitation fluid compared with WB. Other than the biochemical parameters evaluated, LR is cheaper in price and easier for storage. Hence, one would think that resuscitation with LR has more advantages than that with WB in practicality.

In summary, hemorrhage increased caspase-3 activity and decreased ATP levels in rat lung and ileum. LR inhibited the increased caspase-3 activity and restored ATP levels in the lung, and WB inhibited only caspase-3 activity in ileum. The hemorrhage-induced increases in lung were probably mediated by iNOS overexpression that led to increased levels of caspase-3 and perhaps also ATP loss. Resuscitation with LR or WB reversed the adverse effects caused by hemorrhage by downregulating iNOS and its related proteins.

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REFERENCES

Kiang JG, Warke VG, and Tsokos GC.
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