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Ethyl Pyruvate Prevents Inflammatory responses and Organ Damage During in Porcine Hemorrhage

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Abstract

Hemorrhage remains a common cause of death despite the recent advances in critical care, in part because conventional resuscitation fluids fail to prevent lethal inflammatory responses. Here, we analyzed whether ethyl pyruvate can provide a therapeutic anti-inflammatory potential to resuscitation fluids and prevent organ damage in porcine hemorrhage. Adult male Yorkshire swine underwent lethal hemorrhage with trauma and received no resuscitation treatment or resuscitation with Hextend alone, or supplemented with ethyl pyruvate. Resuscitation with ethyl pyruvate did not improve early hemodynamics, but prevented hyperglycemia, the intrinsic coagulation pathway, serum aspartate aminotransferase, and myeloperoxidase in the major organs. Resuscitation with ethyl pyruvate provided an anti-inflammatory potential to restrain serum TNF and HMGB1 levels. Ethyl pyruvate inhibited NF- κ B in the spleen but not in the other major organs. In contrast, ethyl pyruvate inhibited nitric oxide in all the major organs and it also inhibited TNF production in the major organs but in the lung and heart. The most significant effects were found in the terminal ileum where ethyl pyruvate inhibited cytokine production, restrained myeloperoxidase activity, preserved the intestinal epithelium, and prevented the systemic distribution of bacterial endotoxin. Ethyl pyruvate can provide therapeutic anti-inflammatory benefits to modulate splenic NF- κ B, restrain inflammatory responses, and prevent hyperglycemia, the intrinsic coagulation pathway and organ injury in porcine hemorrhage without trauma.

Keywords

Hemorrhage; Resuscitation; ethyl pyruvate; TNF; HMGB1; NF- κ B; coagulopathies

Introduction

Hemorrhage is a leading cause of death despite the recent advances in critical care (1,2). After losing over 50% of the total blood volume, the system is unable to reestablish tissue perfusion and the animals succumb to lethal organ dysfunction. Thus, conventional resuscitation fluids are designed to restore circulatory volume and tissue perfusion. However, resuscitation triggers inflammatory responses that can be more dangerous than the original hemorrhage. Moderate inflammatory responses are beneficial to prevent ischemic damage, but overzealous responses are detrimental and limit the prognosis of the resuscitation. Among the characteristic inflammatory factors produced during resuscitation,

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tumor necrosis factor (TNF), high mobility group B protein-1 (HMGB1), and nitric oxide (NO) contribute to necrosis, tissue damage, cardiovascular shock and multiple organ failure (3–5). Conventional resuscitation fluids such as Ringer's lactate are lactate-supplemented saline solutions that resemble plasma composition (6,7). Novel resuscitation fluids include Hextend, a colloidal plasma volume expander containing 6% hydroxyethyl starch in a lactated balanced saline solution (8). The hetastarch in Hextend creates oncotic pressure, which is normally provided by blood proteins and permits the retention of intravascular fluid. Resuscitation with Hextend requires less intravenous fluid, attenuates organ injury, and provides particular benefits over saline in hypotensive resuscitation (9–11). Recent studies proposed that pyruvate can provide an anti-inflammatory potential to restrain inflammatory responses and antioxidant capacity to scavenge reactive oxygen species (ROS) to protect mammalian cells from cytotoxicity (12,13). Solutions containing pyruvate are beneficial in experimental models of stroke and hemorrhage (13,14). However, the clinical use of pyruvate is hampered by its instability and toxicity (12,15). Aqueous solutions of pyruvate undergo a spontaneous aldol-like condensation reaction to form parapyruvate, which interfere with the tricarboxylic acid cycle, inhibiting mitochondrial respiration (12,16,17). In order to circumvent these problems, recent studies proposed the use of ethyl pyruvate (18,19), a more stable compound that prevents the side effects of pyruvate while providing similar anti-inflammatory benefits. Ethyl pyruvate is classified as GRAS (generally regarded as safe) by the FDA and it has already been used in clinical trials.

Recent studies indicate that ethyl pyruvate inhibits TNF production in human and murine macrophages (19–21). *In vivo*, ethyl pyruvate provides anti-inflammatory benefits in experimental models of septic shock, polymicrobial sepsis, acute pancreatitis, alcohol hepatitis, acute lung injury, burn, radiation damage, myocardial, and mesenteric or hepatic ischemia/reperfusion, extrahepatic biliary obstruction, zymosan-induced multiple organ failure, and hemorrhage (16). In contrast, a recent phase II multicenter clinical trial failed to confirm the anti-inflammatory potential of ethyl pyruvate in patients with cardiopulmonary bypass (22). A potential explanation is that conventional experimental models of hemorrhage do not mimic the clinical settings of trauma, and rodents have different inflammatory and cardiovascular responses as compared to humans. Unlike rodents, swine have an anatomy, physiology, hemodynamic and inflammatory responses closer to that found in humans (23,24). The anti-inflammatory potential of ethyl pyruvate has not been tested in swine. Actually, most studies in swine are performed in splenectomized animals (25) and the organ-specific inflammatory responses to hemorrhage with trauma still uncharacterized. Our hypothesis is that ethyl pyruvate can provide a beneficial anti-inflammatory potential to restrain inflammatory responses and prevent organ damage during resuscitation in porcine hemorrhage. These studies in large animal models are clinically relevant because it is normally difficult to adequately replicate standard intensive care (eg. mechanical ventilation) in rodents. Our study indicates that swine have fundamental differences in their responses to hemorrhage as compared with rodents. To our knowledge, the present study is the first report comparing the organ-specific inflammatory responses in porcine hemorrhage with trauma and the anti-coagulant potential of ethyl pyruvate to prevent coagulopathies. Our results indicate that ethyl pyruvate prevents inflammatory responses and organ injury, yet these two processes have different pattern.

Material and Methods

Animal Experiments

All animal experiments were performed in accordance with the Care and Use of Laboratory Animals Guidelines, and approved by the Institutional Animal Care Committee of the UMDNJ-New Jersey Medical School. Adult male Yorkshire swine ~25Kg were fasted overnight with free access to water. Swine were anesthetized with a single intramuscular

administration of glycoxyiolate (10 µg/kg), ketamine (20 mg/kg) and xylazine (2 mg/kg) similar as described in Senthil & Deitch (26). The animals were kept anesthetized with inhalation of isoflurane 3%. A 5-cm incision was performed in the neck to cannulate the internal jugular vein, which was used for blood withdrawal and administration of the resuscitation fluid. The femoral artery in the right leg was used to monitor the mean arterial blood pressure. After catheter implantation, the incisions were closed by suturing with 4-0 silk. During the surgical procedures, the body temperature was recorded and maintained at 37.5 by using external heating pads. Trauma included the fracture of the left femur using a cylindrical Schermer KR-stunner similar to that described by Crookes et. al. (27). The KR-stunner (Karl Schermer GmbH & Co. KG, Germany) consists of a bolt impactor tip of 85 mm damped by means of a rubber buffer with approximate 61 m/sec speed, and 450 Joules of impact energy using the C-5 cartridges. The stunner was applied directly on top of the left knee to induce tissue trauma and bone damage. Bone damage was confirmed by palpation. Hemorrhage was induced by blood withdrawal to reach a mean arterial blood pressure (MAP) of 20–25 mmHg over 30 min via jugular vein as described by Senthil & Deitch (26). Shock was induced by keeping this MABP by continuous blood withdrawal for 30 min. After the shock phase, the animals to be resuscitated received a total of 15 mL Hextend/kg (equivalent to 1000 mL of Hextend in a 70 kg man) during 30 min at a constant flow administered iv into the internal jugular vein. Shed blood was considered lost and was not reinfused.

Blood and organs analyses

Blood and organ tissues were collected at three hours after the hemorrhage. Since animals with non-resuscitation (NR) did not receive resuscitation, we referred the collection times to post-hemorrhage (after finishing hemorrhage). This time correlated with the beginning of the resuscitation treatment in the treated animals. Blood was collected from the femoral vein and immediately analyzed using an i-Stat blood analyzer (Abbot Laboratories, Illinois) for blood chemistry (EC8+) and clotting time (ACT[celite], ACT[kaolin], PT/INR). The metabolic changes were analyzed by the detection of electrolytes, pH, bicarbonate (HCO₃), base excess of extracellular fluid (BE_{ecf}), anion gap (AnGap), glucose, hematocrit, and hemoglobin, as well as total serum protein (TP). Blood analyses also include total and partial carbon dioxide (TCO₂, PCO₂), and blood urea nitrogen (BUN). Serum was obtained by allowing the blood to clot for 1h at room temperature, and then centrifuged for 5 mins at 1,500 × g. Serum was used to determine protein concentration, aspartate aminotransferase, TNF, and bacterial endotoxin. Protein concentration was analyzed using Bio-Rad protein assay (cat# 500-006, Bio-Rad Lab, Inc. CA). Aspartate aminotransferase was analyzed by ELISA (AST, C164-0A C154-0A, CATACHEM Inc, Bridgeport, CT) following the manufacturer's instructions. TNF concentration was determined by ELISA (R&D Systems, Inc., Minneapolis, MN). Bacterial endotoxin was analyzed using the limulus amoebocyte lysate (LPS, QCL-1000, Cambrex Bio Science Walkersville Inc, MD). The organ tissues were harvested at three hours after the hemorrhagic shock. Organs were homogenized in PBS and the homogenates were cleared by centrifugation at 4°C for 10 mins at 1,000 × g and the homogenates were normalized by total protein concentration. Myeloperoxidase was analyzed using the Kit E80PX (Immunology Consultants Lab Inc, Newberg, OR). The activation of p65NF-κB binding to DNA was analyzed by TransAM DNA-Binding ELISA (Active Motif; Cambridge, MA) using the specific probes provided by the manufacturer. Nitric Oxide (NO₂–/NO₃–) was analyzed in tissue homogenates filtered through a 10000 MWCO device and normalized by protein concentration, following manufacturer's instructions (Assay Kit from Assay Designs, Ann Arbor, MI). Briefly total nitrate/nitrite concentration was determinate in a two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase and the second step is the addition of the Griess

Reagents which convert nitrite into a deep purple compound. Photometric measurement of the absorbance was performed at 540 nm.

Histological analyses

Ileum samples were stained with hematoxylin-eosin. Three hours after the experimental hemorrhage, ileum samples were harvested, fixed in 10% buffered formalin overnight dehydrated with ethanol xylene and embedded in paraffin block. Four- μ m sections were stained with hematoxylin-eosin. Five random fields with 100 villi from each animal were then analyzed using light microscopy at 40 $\times$ and 100 $\times$ magnification. Mucosal damage was scored according with the morphological changes described by Baskett et al. (28): Grade 0: Normal mucosal villi. Grade 1: development of subepithelial Gruenhagen's space, usually at the apex of the villus; often with capillary congestion. Grade 2: Extension of the subepithelial space with moderate lifting of epithelial layer from lamina propria. Grade 3: Massive epithelial lifting down the side of villi. A few tips may be denuded. Grade 4: denuded villi with lamina propria and dilated capillaries exposed. Increased cellularity of lamina propria may be noted. Grade 5: Digestion and disintegration of lamina propria, hemorrhage and ulceration.

Statistical Analyses

Analyses of normality and homogeneity of variance were performed to verify the assumptions of ANOVA. Statistical analyses were performed using the one way ANOVA with the Bonferroni's corrections. *represents $P < 0.05$ vs Control. ** represents $P < 0.01$ vs Control. # represents $P < 0.05$ HEP vs HXT. ## represents $P < 0.01$ HEP vs HXT. $n = 4$ animals/group.

Results

Model Development and Characterization

First, we developed a lethal model of hemorrhage in swine similar to that used to study ethyl pyruvate in rodents (29,30). Rodents were bled for 15 min to reach a mean arterial blood pressure of 35–40 mmHg, and that blood pressure was maintained for another 15 min (29). All hemorrhagic rodents without resuscitation died within the first four hours after hemorrhage (29). Unlike rodents, all hemorrhagic swine without resuscitation survived this procedure ($n=4$). Then, we analyzed the conditions to achieve in swine a mortality pattern similar to that described in rodents. Our final experimental strategy was to bleed the swine for 30min to reach a mean arterial blood pressure of 20–25 mmHg. This blood pressure was maintained for 30 min by continuous blood withdrawal or re-infusion. Similar to rodents, all swine without resuscitation did not reestablish normal physiological blood pressure (Fig. 1A) and died within the first four hours after hemorrhage. A pilot study indicated that all swine resuscitated with Hextend alone or supplemented with ethyl pyruvate survived the first 10 hours after resuscitation ($n=5$ swine/group). Our previous studies indicate that all nonsurvival animals died within the first 6 hours after a similar hemorrhagic shock. All the animals that passed this critical period, survived the hemorrhagic shock, and no late deaths were found even the animals were followed for up to seven days (29). Characteristic pathological markers were assessed by blood chemistry at three hours post-hemorrhage, the latest time point with over 50% survival in all the experimental groups including the control animals without resuscitation treatment. Since the control animal group did not receive resuscitation, we referred all the collection times to post-hemorrhage (after finishing hemorrhage). Hemorrhagic swine without resuscitation were characterized by azotemia, metabolic acidosis and hyperglycemia (Fig. 1). This experimental hemorrhage did not induce a statistical effect on packed cell volume (PCV), hemoglobin, total or partial CO_2 , or characteristic electrolytes (sodium, chloride) (data not shown). Resuscitation with Hextend

alone or supplemented with ethyl pyruvate reestablished blood pressure with a similar kinetic; and there were not statistic significant different between both treatments (Fig. 1A). Neither resuscitation with Hextend nor ethyl pyruvate prevented azotemia (Fig. 1B). Both, resuscitation with Hextend alone or supplemented with ethyl pyruvate improved metabolic acidosis, anion gap and negative base excess of extracellular fluid (Fig. 1C–E). These effects on metabolic acidosis did not correlate with changes in bicarbonate, gases (total and partial CO₂), or characteristic electrolytes (sodium, chloride) (data not shown). Neither hemorrhage nor resuscitation with Hextend or ethyl pyruvate affected serum bicarbonate levels in any of the experimental groups (Fig. 1F). One of the most significant effects of hemorrhage and ethyl pyruvate was found in hyperglycemia. Hemorrhage induced a hyperglycemic responses (Fig. 1G) that were not prevented by resuscitation with Hextend. In contrast, resuscitation with ethyl pyruvate significantly prevented these responses and decreased glucose levels toward normal values. The only statistically significant difference between the resuscitation with Hextend alone or supplemented with ethyl pyruvate was the potential of the last to prevent hyperglycemia (Fig. 1G).

Ethyl Pyruvate attenuated blood clotting time and prevented organ damage during resuscitation

The effects of ethyl pyruvate in the intrinsic and extrinsic coagulation pathways were analyzed using the celite, kaolin, activated clotting time, prothrombin time, and the international normalized ratio (Fig. 2). Hemorrhage induced a significant activation of the intrinsic pathway as determined by the celite and kaolin clotting time. Both intrinsic activity tests measure thrombin activation of coagulation cascade, but have slightly different anti-coagulant ranges and sensitivities to protease inhibitors. Hemorrhage increased both the celite and kaolin clotting times in a similar fashion. However, celite-ACT tests provided more reproducible data with lower standard deviation giving more statistically significant results. Resuscitation with Hextend failed to prevent this process, and these animals had activated clotting times significantly higher than control animals. In contrast, ethyl pyruvate significantly prevented the activation of the intrinsic pathway, toward levels statistically similar to that in control animals. All groups showed similar extrinsic clotting as determined by prothrombin clotting time and international normalized ratio, and these times were not significantly affected by either hemorrhage or resuscitation. Organ damage was analyzed by aspartate aminotransferase and myeloperoxidase activities. Hemorrhage significantly increased aspartate aminotransferase activities in the serum. Unlike Hextend, ethyl pyruvate statistically prevented serum aspartate aminotransferase activity (Fig. 3A), toward values statistically similar to those in control animals. Hemorrhage significantly increased myeloperoxidase activity in all the organs (Fig. 3B–F). The most significant induction of myeloperoxidase was found in the heart, liver and terminal ileum. Resuscitation with Hextend alone failed to prevent myeloperoxidase levels in any organ. Resuscitation with ethyl pyruvate prevented myeloperoxidase levels in the lung, heart and terminal ileum but not in the spleen and liver. The most significant effects were found in the terminal ileum, where ethyl pyruvate inhibited myeloperoxidase levels by 40% as compared to those animals resuscitated with Hextend.

Ethyl pyruvate prevented inflammatory responses during resuscitation

Previous studies indicated that serum TNF peaks at approximately three hours after hemorrhage in both rodents and swine (29,31). Control animals have significant serum TNF levels induced by the sham surgery as compared with the physiological values of serum TNF before the sham surgery of <50 pg/mL (Fig. 4A). Hemorrhagic animals without resuscitation had over 2-fold higher serum TNF levels than the control animals. Resuscitation with Hextend alone failed to attenuate serum TNF levels. However, Hextend supplemented with 50mM ethyl pyruvate significantly decreased serum TNF levels by

~60% as compared to Hextend alone. Serum TNF levels in the animals resuscitated with ethyl pyruvate were statistically similar to those in the control animals. Hemorrhage also induces serum HMGB1 levels and resuscitation with Hextend alone or supplemented with ethyl pyruvate significantly attenuated serum HMGB1 levels. Yet, ethyl pyruvate was significantly more efficient at inhibiting serum HMGB1 as compared with Hextend (Fig. 4B). Hemorrhage did not significantly increase TNF levels in the lung or the heart as compared to that in the sham animals (Fig. 4C,D), but it increased TNF levels in the spleen, liver, and terminal ileum by ~2-fold, a pattern similar to that found in the serum (Fig. 4E–G). The largest induction of TNF was found in the terminal ileum, where hemorrhage increased TNF levels by over 2.5-fold. Resuscitation with Hextend failed to inhibit TNF levels in any organ. In contrast, ethyl pyruvate significantly inhibited TNF levels in the spleen, liver and terminal ileum toward values statistically similar to those in the control animals. Similar results were found, when TNF RNA levels were analyzed by real-time PCR (data not shown). In contrast, neither hemorrhage nor resuscitation with ethyl pyruvate affected TNF levels in the lung or heart. Since p65NF- κ B modulates TNF production, we analyzed its implications in the anti-inflammatory potential of ethyl pyruvate. Hemorrhage significantly activated p65NF- κ B in all the organs but in the lung. Again, the most significant activation of this pathway was found in the terminal ileum with over 2-fold of induction as compared with control animals (Fig. 5E). Resuscitation with Hextend alone inhibited the p65NF- κ B in the heart, liver and terminal ileum, but not in the spleen. In contrast to Hextend, ethyl pyruvate was particularly efficient inhibiting p65NF- κ B in the spleen. The benefits of ethyl pyruvate modulating myeloperoxidase in the heart and lung did not correlate with TNF or p65NF- κ B inhibition. The inhibition of TNF in the liver and ileum did not correlate with p65NF- κ B modulation. Then, we analyzed whether ethyl pyruvate could provide benefits in the terminal ileum by modulating nitric oxide (NO). Hemorrhage increased NO levels in all the major organs, being particularly significant in the terminal ileum (Figure 6A–E). Resuscitation with Hextend failed to prevent NO levels in any organ but in the liver. In contrast, ethyl pyruvate prevented NO levels in all the organs as compared to resuscitation with Hextend toward values statistically similar to that found in control animals. Again, the most significant effects were found in the terminal ileum. While hemorrhage increases NO levels by 3-fold as compared with sham surgery (control), ethyl pyruvate completely prevented this process.

Ethyl pyruvate preserved intestinal epithelium, and prevented bacterial leakage

Since the most significant effects of ethyl pyruvate related with the terminal ileum, we analyzed the intestinal epithelium and potential bacterial leakage. Sections from the terminal ileum were collected and stained for Hemotoxylin-Eosin histology (Fig. 5). The intact lamina propria of control sham animals showed normal intestinal morphology with the characteristic epithelial distribution (Grade 0). Animals without resuscitation had denuded villi with increased cellularity, dilated capillaries, and lamina propria exposed (Grade 4). Resuscitation with Hextend alone induced massive epithelial lifting in approximately 40% of the ileum surface down the side of the villi and over 75% of denuded apex (Grade 3), diminution of villous height and gut mucosal goblet cell number. Resuscitation with Hextend supplemented with ethyl pyruvate induced a dramatic effect by completely preventing epithelial lifting and denuded villi. Moreover, animals treated with ethyl pyruvate showed minor development of subepithelial Gruenhagen's space usually at the apex of some villi (Grade 1), but normal villous height and mucosal goblet cell number. These results suggest that resuscitation with ethyl pyruvate can preserve intestinal epithelium and prevent bacterial leakage. Bacterial endotoxin was measured in the blood collected at seven hours post-hemorrhage by using the Limulus Amebocyte Lysate test (Fig. 5E). Hemorrhage disrupted intestinal morphology, which correlated with a significant increase of bacterial endotoxin in the blood. Resuscitation with Hextend did not prevent these levels of serum

LPS. However, resuscitation with ethyl pyruvate preserved intestinal morphology and significantly prevented serum LPS levels as compared with those animals resuscitated with Hextend. Animals resuscitated with ethyl pyruvate had serum bacterial endotoxin levels statistically similar to that found in control animals. The serum bacterial endotoxin levels for all samples correlated with the integrity of the intestinal epithelium.

Discussion

Despite the large number of studies claiming the benefits of ethyl pyruvate in rodents with myocardial ischemia/reperfusion, hemorrhagic or septic shock, and multiple organ injury; clinical trials have failed to confirm these effects of ethyl pyruvate in cardiopulmonary bypass (16). A potential explanation is that conventional experimental models of hemorrhage do not mimic the clinical settings of trauma, and rodents have different inflammatory and cardiovascular responses as compared to humans. Swine closely resemble human anatomy, cardiovascular hemodynamics, physiology and inflammatory responses (23). Indeed, our study indicates that swine were more resistant to lethal hemorrhage as compared to rodents (29,30). To date, only two studies analyzed the effects of ethyl pyruvate in swine. The first study indicated that ethyl pyruvate improved hemodynamics and organ function in porcine *endotoxemia* (32), similar to previously reported in rodents (18). In the second study, ethyl pyruvate failed to improve hemodynamics in *splenectomized* swine with hemorrhage (25). In our present study, ethyl pyruvate also failed to provide hemodynamic benefits in nonsplenectomized swine, yet it prevented organ damage. These results concur with the phase II clinical trial where ethyl pyruvate failed to provide hemodynamic benefits in cardiopulmonary bypass (22). The clinical implications of our study are determined by four considerations. First, unlike previous studies using sublethal conditions in swine, our experimental model is a lethal hemorrhage. Similar to clinical standards where the patients die quickly or survive long-term, all control swine without resuscitation died within the first hours after hemorrhage. Second, our studies used Hextend as resuscitation fluid. Resuscitation with Hextend requires less intravenous fluid, attenuated organ injury, and provided significant survival benefits over saline in hypotensive resuscitation (9–11). Third, similar to clinical settings of trauma, shed blood was considered lost and was not reinfused. Animals were treated with a small resuscitation volume of 15mL/Kg Hextend fluid that represents <40% of the total shed blood volume. Studies with limited resuscitation fluid are particularly relevant to clinical settings with limited supplies, long times of evacuation, or severe trauma (11,33). In addition, other studies indicated that normotensive resuscitation with high volume can increase hemorrhage volume and mortality when shock is associated with major vascular injury (34). Fourth, unlike classical experimental models, hemorrhage in critical care is normally associated with collateral trauma that affects the physiological and inflammatory responses to hemorrhage and resuscitation. Our experimental models include trauma induced by broken femur and soft tissue injury. Of note, our porcine model does not include splenectomy, a common approach in other porcine studies. This observation is a significant consideration because the spleen contributes to the inflammatory responses during resuscitation and trauma. Our previous studies indicate that splenectomy attenuated serum TNF levels in experimental sepsis (35). However, splenectomy changed the inflammatory responses but failed to attenuate serum TNF levels in experimental hemorrhage without trauma (29). To our knowledge, the present report is the first study comparing the organ-specific inflammatory responses in porcine hemorrhage with trauma. Our studies indicate that the inflammatory responses in swine are most sensitive than in rodents. Although swine and rodents are characterized by similar serum TNF levels (~300 pg/mL), their pathophysiological responses to hemorrhage are different and particularly in the lung and the heart. Hemorrhage in the rats causes dramatic TNF increases in all the organs including the lung and heart (75–100 TNF pg/ng protein). However, porcine hemorrhage with trauma is characterized by lower TNF responses in most of the organs but

particularly in the lung and heart (20–40 TNF pg/ng protein). Together, these results indicate that TNF responses in the lung and heart are fundamentally different in swine and rodents, and they may contribute to the higher resistance of swine to lethal hemorrhage. In addition, ethyl pyruvate inhibited TNF production in most of the organs but not in the lung or heart during resuscitation in experimental hemorrhage with trauma. It could be possible to propose that the inability of ethyl pyruvate to control TNF levels in the lung and heart might have prevented its anti-inflammatory potential in the clinical trials of cardiopulmonary bypass (22). Yet, these results warrant future studies about the molecular mechanisms of ethyl pyruvate in different organs and its potential implications for the treatment of multiple organ failure in trauma.

The regulation of TNF in different organs by ethyl pyruvate did not correlate with their myeloperoxidase responses. Ethyl pyruvate inhibited TNF levels in the spleen and liver, yet it did not prevent myeloperoxidase activity in these organs. In contrast, ethyl pyruvate did not inhibit TNF responses in the lung and heart, and yet it prevented their myeloperoxidase responses. Previous studies suggested that ethyl pyruvate controls systemic inflammation by inhibiting TNF production in macrophages. A recent study indicates that both ethyl pyruvate and ethyl lactate can decrease cytokine production in human monocytes. Of note, ethyl pyruvate appears more efficient inhibiting LPS-induced TNF production whereas ethyl lactate appears more efficient inhibiting IL-8 (36). These results may suggest different mechanisms, yet future studies will be required to confirm this hypothesis. Ethyl pyruvate restrains cytokine production in macrophages by inhibiting p65NF- κ B, a characteristic modulator of TNF production. We reported that ethyl pyruvate inhibits LPS-induced activation of p65NF- κ B in RAW macrophages cells (19). Similar studies in HEK293 cells indicated that ethyl pyruvate inhibits DNA-binding of wild-type p65NF- κ B but not the mutant form of p65NF- κ B with substitution of serine for cysteine 38 (37). These results suggest that ethyl pyruvate may inhibit DNA-binding by covalently modifying p65NF- κ B at Cys38 (37). Unexpectedly, our present study indicates that ethyl pyruvate failed to inhibit p65NF- κ B in most of the organs but in the spleen. These results suggest that the mechanism of ethyl pyruvate *in vivo* is more complicated than proposed in particular cell lines, and organ-specific studies can provide novel insights on its therapeutic potentials. Previous studies reported that ethyl pyruvate prevented arterial nitrate/nitrite levels during endotoxemia (32) and iNOS induction in Caco-2 cells treated with inflammatory factors (38). Our present results also indicate that ethyl pyruvate inhibited NO in all the organs analyzed. Previous studies have already shown the implications of NO and peroxynitrite in organ damage during resuscitation. NO donors, including sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, and 3-morpholinodimethylamine increase the permeability of Caco-2 monolayers. The hyperpermeability of Caco-2 cells treated with inflammatory cytokines is prevented by pharmacological agents that inhibit iNOS or scavenge NO (38). In this direction, iNOS^{-/-} knockout mice are resistant to intestinal derangement and bacterial translocation during *endotoxemia* (39). Since NO modulation prevents the oxidative potential of peroxynitrite, these results provide a link between the anti-inflammatory and the anti-oxidant potential of ethyl pyruvate. These results concur with our study showing the most significant effects of ethyl pyruvate in the terminal ileum. Unlike Hextend, ethyl pyruvate prevented intestinal NO, TNF, and myeloperoxidase levels, preserved the intestinal epithelium, and prevented serum bacterial endotoxin. Intestinal derangement correlated with the presence of bacterial endotoxin in the serum, suggesting that ethyl pyruvate may prevent the leakage of the intestinal bacteria and thereby the susceptibility to septicemia during resuscitation. These results concur with recent studies indicating that TNF mediates hyperglycemia-augmented intestinal barrier dysfunction (40). Unlike previously reported in rodents, our study indicates that ethyl pyruvate prevented hyperglycemia in porcine hemorrhage. Previous studies reported that pyruvate is not insulinogenic (41), but methyl pyruvate stimulates insulin secretion in isolated pancreatic islets (42). It was proposed that

methyl pyruvate is more lipophilic and it might penetrate the mitochondrial matrix and support ATP production. Yet, other studies indicated that methyl pyruvate initiates membrane depolarization and insulin release by metabolic factors other than mitochondrial ATP production (43). Similar studies will be required to study the effects of ethyl pyruvate in pancreatic islets. However, since hyperglycemia can enhance cytokine production (44), it is tempting to suggest that the antiglycemic potential of ethyl pyruvate may contribute to its anti-inflammatory and anti-coagulant benefits in porcine hemorrhage. Indeed, similar to our present study, ethyl pyruvate also failed to affect the prothrombin time in the clinical trial (22). However, we found that ethyl pyruvate efficiently inhibited the intrinsic pathway as determined by the celite and kaolin clotting time. These results concur with previous studies *in vitro* indicating that ethyl pyruvate prevents the production of pro-coagulant factors from human monocyte-like THP1 cells activated with LPS (16,45). To our knowledge, our present study is the first evidence *in vivo* of the anti-coagulant potential of ethyl pyruvate to prevent coagulopathies. These results warrant future studies on ethyl pyruvate to preserve organ function during resuscitation and for the potential treatment of coagulopathies mediated by the intrinsic pathway. In summary, our current study suggests that porcine hemorrhage can provide significant advantages to study the clinical limitations of ethyl pyruvate. Indeed, the effects of ethyl pyruvate in porcine hemorrhage are significantly different to that described in rodents. Ethyl pyruvate can provide therapeutic anti-inflammatory benefits to modulate splenic NF- κ B, restrain inflammatory responses, and prevent hyperglycemia, the intrinsic coagulation pathway and organ injury during resuscitation in lethal porcine hemorrhage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. MABP and Blood chemistry analyses

Control Adult male Yorkshire swine were subjected to sham surgery (control), hemorrhage without resuscitation (NR), or hemorrhage and resuscitation with 15mL/Kg (iv) Hextend (HXT) or Hextend supplemented with 50 mM ethyl pyruvate (HEP). (A) Arterial blood pressure was recorded during the hemorrhage and the resuscitation treatment. Blood was analyzed for (B) blood urea nitrogen (BUN), (C) pH, (D) the anion gap (AnGap), (E) base excess of extracellular fluid (BEecf), (F) bicarbonate (HCO₃) and (G) Glucose.

Figure 2. Ethyl pyruvate decreased the intrinsic coagulation pathway

Control (C) or hemorrhagic Yorkshire swine without resuscitation (NR), or treated with Hextend alone (HXT), or Hextend supplemented with ethyl pyruvate (HEP). Blood coagulation tendency was determined by analyzing the intrinsic: (A) celite Activated Clotting Time (cACT), (B) kaolin Activated Clotting Time (kACT), and the extrinsic coagulation pathway: (C) prothrombin time (PT), and (D) the international normalized ratio (INR).

Figure 3. Resuscitation with ethyl pyruvate prevented organ damage in porcine hemorrhage Control (C) or hemorrhagic Yorkshire swine without resuscitation (NR), or resuscitated with Hextend alone (HXT), or Hextend supplemented with ethyl pyruvate (HEP). Serum and organs were analyzed at three hours after hemorrhage for (A) aspartate aminotransferase (AST) activity in serum, and for myeloperoxidase (MPO) activity in (B) lung, (C) heart, (D) spleen, (E) liver, and (F) terminal ileum

Figure 4. Resuscitation with ethyl pyruvate restrained inflammatory responses during resuscitation

Control (C) or hemorrhagic Yorkshire swine without resuscitation (NR), or resuscitated with Hextend alone (HXT), or with ethyl pyruvate (HEP) were euthanized at three hours after hemorrhage to analyze serum (A) TNF and (B) HMGB1 as well as TNF in the major organs including (C) lung, (D) heart, (E) spleen, (F) liver, and (G) terminal ileum.

Figure 5. Ethyl pyruvate modulated p65NF-κB during resuscitation

Control (C) or hemorrhagic Yorkshire swine without resuscitation (NR), or resuscitated with Hextend alone (HXT), or with ethyl pyruvate (HEP) were euthanized at three hours after hemorrhage to analyze the NF-κB pathway in the (A) lung, (B) heart, (C) spleen, (D) liver, and (E) terminal ileum. The graph represents the relative activity (r.a.) of the p65NF-κB binding to specific probe and normalized according to the control

Figure 6. Ethyl pyruvate modulated nitric oxide during resuscitation

Control (C) or hemorrhagic Yorkshire swine without resuscitation (NR), or resuscitated with Hextend alone (HXT), or with ethyl pyruvate (HEP) were euthanized at three hours after hemorrhage to analyze the nitrate/nitrite (NO_3/NO_2) in the (A) lung, (B) heart, (C) spleen, (D) liver, and (E) terminal ileum.

Figure 7. Ethyl pyruvate preserved intestinal epithelium, and prevented serum bacterial endotoxin

Representative sections of ileum were taken for histological examination. Control (C), or hemorrhagic swine without resuscitation (NR), or treated with Hextend alone (HXT), or Hextend supplemented with ethyl pyruvate (HEP) were stained by Hematoxylin-Eosin. Hemorrhagic animals treated with Hextend showed massive epithelial lifting and denuded villi that was prevented ethyl pyruvate. Two sections from different animals treated with HXT are shown. (E) Bacterial endotoxin (LPS) levels in serum were measured in control (C) or hemorrhagic swine without resuscitation (NR) or treated with Hextend alone (HXT) or supplemented with ethyl pyruvate.