The Effect of Inhibition of a Major Cell Signaling Pathway Following Trauma Hemorrhage on Hepatic Injury and Interleukin 6 Levels

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Hypothesis: Recent studies have shown that intracellular signaling pathways, such as the mitogen-activated protein kinases, play a pivotal role in the activation of the inflammatory response. We hypothesized that administration of a specific mitogen-activated protein kinase inhibitor, PD 98059, at the end of resuscitation following severe hemorrhagic shock can reduce the plasma levels of interleukin 6 (IL-6) and hepatocellular damage.

Design: Prospective controlled animal study.

Setting: Medical school–affiliated university hospital.

Interventions: Male Sprague-Dawley (275-325 g) rats underwent laparotomy (ie, soft tissue trauma) and were then bled to a mean arterial pressure of 40 mm Hg for approximately 90 minutes. The animals were then resuscitated with 4 times the bleed-out volume using Ringer lactate solution for 60 minutes. PD 98059, an inhibitor of extracellular signal–regulated kinases (ERKs) 1 and 2 (750 mmol/L), or vehicle (dimethyl sulfoxide and isotonic sodium chloride solution) was administered intravenously as a bolus at the end of resuscitation.

Main Outcome Measures: At 24 hours after resuscitation or sham operation, plasma levels of IL-6 and α-glutathione S-transferase were determined with enzyme-linked immunosorbent assay and enzyme immunoassay, respectively. Moreover liver sections were stained with monoclonal antibody against the phosphorylated form of ERKs.

Results: At 24 hours following trauma hemorrhage and resuscitation, plasma levels of IL-6 and α-glutathione S-transferase were markedly elevated. Administration of PD 98059, however, reduced levels to sham values. Moreover, liver expression of phosphorylated ERKs was found in the cytosol and nuclear compartment of hepatocytes only following trauma hemorrhage.

Conclusion: Administration of PD 98059 (ie, inhibition of intracellular signaling pathways) may represent a feasible approach to blunt the inflammatory response and improve outcome following traumatic injuries and hemorrhagic shock.

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TRAUMA AND ITS SEQUELAE, such as multiple organ failure, remain a leading cause of morbidity and mortality in the younger population despite advances inprehospital care and improved diagnostic modalities. Death usually occurs in a biphasic manner, with an early peak due to the severity of the insult (ie, exsanguination and brain injury) and a late peak due to multiple organ failure and septic complications.1 Although resuscitation with crystalloids and blood transfusion remain the cornerstone in the initial treatment of hemorrhagic shock, maintenance of intravascular volume and adequate perfusion does not prevent the onset of the systemic inflammatory response syndrome (SIRS).2,3

Experimental and clinical studies have indicated that the early inflammatory response to low-flow conditions and regional hypoxia, such as soft tissue trauma and subsequent blood loss from a severed blood vessel, perpetuates and causes cell and organ dysfunction once a certain threshold has been reached. Several clinical studies4-6 have shown that SIRS, exemplified by elevated plasma levels of interleukin 6 (IL-6), correlates with the magnitude of the insult and can be used to predict mortality. Moreover, it seems that activation of intracellular signaling cascades, such as the mitogen-activated protein kinases (MAPKs), are responsible for relaying and coordinating the environmental stress signals to the genome, with subsequent alteration in gene expression.7,10

Recent studies12,13 have shown that α-glutathione S-transferase (α-GST) is a sensitive indicator of hepatocyte damage following adverse circulatory conditions.
In 2 studies, levels of α-GST were elevated earlier than the elevation in liver transaminase levels. Because the half-life time of α-GST is less than an hour, it serves as an excellent marker not only for detecting liver damage but also for assessing the efficacy of therapeutic interventions. The levels of prostaglandin E₂ (PGE₂) were measured, because recent evidence suggests that PD 98059 not only may inhibit extracellular signal regulated kinase (ERK) phosphorylation but also may block cyclooxygenase 2 (COX-2) activity above certain concentrations. COX-2 is also responsible for the production of PGE₂, which acts as an immunosuppressive mediator. Thus, no changes in the plasma levels of PGE₂ would indicate that the beneficial effects of PD 98059 are more likely to be due to inhibition of the ERK pathway.

With the recent availability of MAPK inhibitors, the roles of these signaling pathways following adverse circulatory conditions can be elucidated in more detail. The MAPK family has received increased attention, because its activation is attributed specifically to the stress response. Among the 4 distinct subgroups are the ERKs (p44/42), c-Jun N-terminal or stress-activated protein kinases, ERK 5, and the p38 group (Figure 1). Koj et al have shown that ERK (p44/42) plays an important role in initiating the hepatic acute-phase response during inflammatory conditions. These kinases phosphorylate, usually in a multistep cascade, transcription factors that belong primarily to CCAAT enhancer binding protein (C/EBP), nuclear factor κB, and activator protein 1 families, which then regulate a variety of genes, such as inflammatory cytokines and the acute-phase response. The aim of this study, therefore, was to determine the effects of the MAPK ERK (p44/42) inhibitor PD 98059 on the systemic inflammatory response by measuring plasma levels of IL-6 and hepatic damage as evidenced by liver release of α-GST.

**METHODS**

**EXPERIMENTAL PROCEDURES**

Our previously described nonheparinized model of trauma hemorrhage in the rat was used with minor modifications. Briefly, male Sprague-Dawley rats (275-325 g; Charles River Laboratories, Wilmington, Mass) were fasted overnight before the experiment but allowed water ad libitum. The rats were anesthetized by methoxyflurane (Mallinckrodt Veterinary Inc, Mundelein, Ill) inhalation before the induction of trauma (ie, 5-cm midline laparotomy). The abdomen was then closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (polyethylene tubing, PE-50; Becton Dickinson & Co, Sparks, Md). The wounds were bathed with 1% lidocaine (Eliks-Sinn Inc, Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. Rats were allowed to awaken and then bled to maintain a mean arterial pressure (MAP) of 40 mm Hg. This level of hypotension was continued until the animals could not maintain a MAP of 40 mm Hg unless extra fluid in the form of Ringer lactate solution; at this level, the rats were allowed water ad libitum. The rats were then resuscitated with 4 times the volume of the withdrawn blood over 60 minutes with Ringer lactate solution. At the end of resuscitation, animals received a bolus (approximately 0.8 mL) of the ERK inhibitor PD 98059 (750 mmol/L; Calbiochem, Anahein, Calif) or an equal volume (approximately 0.8 mL) of the vehicle isotonic sodium chloride–dimethyl sulfoxide (DMSO) solution. The dose of PD 98059 was derived from previous studies by Talarmin et al. The shed blood was not used for resuscitation. Animals in the sham operation group underwent the same groin dissection, which included the ligation of the femoral artery and vein; however, neither hemorrhage nor resuscitation was performed.

At 24 hours following the completion of fluid resuscitation or sham operation, the animals were anesthetized with methoxyflurane, and serum samples were obtained. The animals were humanely killed using ketamine hydrochloride, and liver samples from different lobes were obtained and immediately snap frozen in liquid nitrogen.

All animal experiments were performed according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health, Bethesda, Md. This project was approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham.

**SERUM IL-6 LEVELS**

Twenty-four hours after resuscitation, whole blood was obtained and placed in microcentrifuge tubes. The tubes were then centrifuged at 14,000g for 15 minutes at 4°C. Serum was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored at −70°C until assayed. The IL-6 levels were measured using an enzyme-linked immunosorbent assay kit (BioSource International, Camarillo, Calif) according to the manufacturer’s instruction.

**SERUM α-GST MEASUREMENT**

Plasma levels of α-GST were quantified using a rat α-GST enzyme immunoassay according to the manufacturer’s instruc-
Liver sections from animals in the sham operation group showed no staining for the phosphorylated form of p44/42. In contrast, abundant staining was found in the cytosolic and nuclear compartment of hepatocytes in animals undergoing the trauma hemorrhage procedure and vehicle treatment (Figure 2). However, liver sections from PD 98059–treated animals showed no such staining.

**COMMENT**

Studies have shown that organ dysfunction and subsequent multiple organ failure are common complications in survivors of trauma-induced hypotension.22 The lung, kidney, and liver are among the most frequently affected organs. Several studies have shown that the rise in plasma levels of IL-6 is uniquely consistent and correlates with the inflammatory response following adverse circulatory conditions. However, it remains unclear whether IL-6 is indeed the culprit for producing organ failure following severe injury or whether it is just a valuable biochemical marker of the inflammatory response. Nonetheless, early postinjury IL-6 levels predict the incidence of complications and correlate with mortality.5 Studies by Partrick et al7 and Biffl et al8 have shown that plasma levels of IL-6 predict the incidence of multiple organ failure in trauma patients, whereas other cytokines, such as tumor necrosis factor α, are less predictive of outcome.

Although a magnitude of cell types are capable of producing IL-6, monocytes, alveolar macrophages, and Kupffer cells (sesile macrophages) appear to be the most relevant cell populations. In this regard, O’Neill et al23 have shown that blockade of Kupffer cells with gadolinium chloride before trauma hemorrhage inhibits IL-6 release following trauma hemorrhage. Thus, it appears that the liver with its parenchymal cells and Kupffer cells is responsible for the systemic release of IL-6 following low-flow conditions.

Recent advances in molecular biology and a better understanding of cell signaling have led to the detailed description of intracellular signaling pathways. Among those, the MAPK family plays an important role in relaying environmental stress signals to the level of the cytosol and nucleus. Specifically, the members of the ERKs with a molecular weight of 44 and 42 kDa are associated with the inflammatory response and the hepatic acute phase. The aim of our study, therefore, was to determine whether administration of the ERK inhibitor PD 98059 intravenously at the end of crystalloid resuscitation following severe hemorrhagic shock decreases circulating levels of IL-6 and reduces liver damage as assessed by plasma levels of α-GST.

Our results indicate that at 24 hours following trauma hemorrhage and crystalloid resuscitation, plasma levels of IL-6 were significantly elevated compared with animals in the sham operation group. Moreover, levels of α-GST, indicative of hepatocellular damage, were also increased at 24 hours following injury. Immunohistochemi-
analysis of liver sections with an antibody against the phosphorylated form of p44/42 showed staining in the cytosol and nuclear compartment of hepatocytes but not other nonparenchymal cells.

Intravenous administration of PD 98059 as an adjunct at the end of crystalloid resuscitation following hemorrhagic shock decreased plasma levels of IL-6 comparable to animals that underwent sham operation. Moreover, circulating levels of α-GST were markedly reduced at 24 hours following hemorrhagic shock in animals treated with PD 98059. Phosphorylation of liver sections following treatment with PD 98059 was also not evident compared with vehicle-treated hemorrhaged animals.

Because recent evidence suggests that PD 98059 not only blocks the p44/42 kinase complex but also interferes with prostaglandin production via inhibition of the enzyme COX-2 (Figure 1), we measured serum levels of PGE$_2$ in hemorrhaged animals and animals that underwent sham operation. The levels of PGE$_2$ were comparable in all 3 groups, with no statistical difference, suggesting that the effects observed following treatment were not due to any alterations in PGE$_2$ production.

Before the trauma hemorrhage experiments, we conducted preliminary studies to determine whether or not intravenous administration of PD 98059 and its vehicle DMSO–isotonic sodium chloride solution can be performed safely. Intravenous administration of DMSO–isotonic sodium chloride solution and PD 98059 dissolved in DMSO–isotonic sodium chloride solution did not affect blood pressure or heart rate, and animals in the sham operation group remained hemodynamically stable for more than 90 minutes, at which point the animals were humanely killed. Although MAPK inhibitors have been given intraperitoneally or subcutaneously, only a limited number of investigators have used such agents intravenously. Our study indicates that MAPK inhibitors can be safely infused intravenously following trauma hemorrhage and that such an inhibitor produces salutary effects under those conditions. This route of administration following trauma hemorrhage is preferable, because it appears that following hemorrhage, perfusion of the skin or the peritoneal cavity is compromised and, therefore, not a reliable route of administration because of questionable bioavailability of the inhibitor.

Other studies have shown that in patients with blunt and penetrating trauma, levels of IL-6 higher than 500 pg/mL predicted subsequent organ failure. However, it remains less clear whether IL-6 is indeed the culprit for the development of multiple organ failure or just the tip of the iceberg with regard to SIRS. Nevertheless, a recent study by Meng et al showed that IL-6 knockout mice had a markedly reduced inflammatory response, such as reduced polymorphonuclear leukocyte activation in lung tissue and a normal pattern of nuclear factor κB in liver tissue compared with wild-type mice following hemorrhagic shock. Although no study has used anti–IL-6 treatment in a model of hemorrhagic shock, such an approach has been successfully used in a model of sepsis. In our rat model of soft tissue trauma and hemorrhage, studies using the earth metal gadolinium chloride to deplete Kupffer cells suggest that this cell population is a main source of circulating IL-6. In this regard, experiments using macrophages and monocytes in cell cultures have shown that following exogenous stimulation with lipopolysaccharide, p44/42 is necessary for the secretion of not only IL-6 but also other proinflammatory mediators such as tumor necrosis factor α or nitric oxide, indicating that the immunomodu-
The inflammatory effects of PD 98059 are likely to extend beyond the reduction of IL-6 release.

Parenchymal liver cells (i.e., hepatocytes) are responsible for the subsequent acute-phase response following stimulation by adjacent Kupffer cells in a paracrine mechanism. The fact that we did not find staining of Kupffer cells for p44/42 except for hepatocytes could be explained by the fact that at 24 hours after resuscitation, the initial surge in cytokine release had subsided and the hepatic acute-phase response was predominant. Therefore, additional studies that focus on the early phase following trauma hemorrhage are needed to better understand the exact mechanism of the beneficial effects of the MAPK antagonist in the treatment of trauma and hemorrhagic shock. Nonetheless, based on our results and published literature, it could be speculated that PD 98059 blunts the inflammatory response following hemorrhagic shock by inhibiting the release of proinflammatory cytokines from Kupffer cells and, thus, reducing the hepatic inflammation and preventing the sustained increase in IL-6 levels. As a consequence, at 24 hours not only were circulating IL-6 levels reduced but also hepatocellular damage and the hepatic acute-phase response were blunted by inhibiting MAPK signaling.

We have not performed studies to determine whether administration of MAPK inhibitor following trauma hemorrhage has any salutary effects on decreasing the lethality from trauma hemorrhage and subsequent sepsis. However, because PD 98059 treatment following trauma hemorrhage normalized all of the measured variables, it seems that such animals should have decreased mortality from trauma hemorrhage and decreased mortality from subsequent sepsis. However, whether this occurs remains to be determined.

We used the standard hospital Ringer lactate solution in our experiments containing a mixture of D-(−) and L-(+ ) isomers. There has been the contention that the D-isomer may enhance the inflammatory response. Although data by Koustova et al.39 showed that the DL-isomer mixture enhances human neutrophil reactive oxygen production compared with the L-isomer, the racemic mixture has been safely used for decades in resuscitation from major surgery and trauma. Moreover, because both trauma hemorrhage groups in our study received the same resuscitation solution, it is more likely that the difference of IL-6 production is due to the ERK inhibitor than to the Ringer lactate solution used.

In summary, our results indicate that hemorrhage-induced release of IL-6 and α-GST was markedly attenuated by the administration of the p44/42 inhibitor PD 98059 at the end of resuscitation. It therefore seems that ERK signaling in liver cells, such as hepatocytes and Kupffer cells, might be responsible for or contribute significantly to liver damage and SIRS following trauma and severe hemorrhage. We conclude that adjuncts to fluid resuscitation that block or attenuate the MAPK signaling cascade seem to be useful for improving organ function following trauma and hemorrhage.

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Statement of Clinical Relevance

Although several clinical studies have shown that elevated levels of IL-6 correlate with an increased rate of postresuscitation complications, it remains unclear whether plasma IL-6 levels are just an epiphenomenon or if IL-6 indeed is the culprit. The data from animal studies support the hypothesis that IL-6 perpetuates the inflammatory response following trauma hemorrhage and participates in causing organ damage such as liver injury. In light of these data findings, it seems rational to use adjuncts to the standard trauma resuscitation to block IL-6. However, IL-6 production and release may be compartmentalized, and plasma levels may not truly reflect the actual tissue levels. For example, portal vein levels of IL-6 may be elevated (originating from the intestine) despite normal systemic levels and cause liver injury. Thus, approaches that block intracellular production of inflammatory mediators may be more successful than neutralizing circulating cytokines.

The recent elucidation of the MAPK system has given us the ability to block specific pathways, with less interference of other signaling pathways. The p44/42 MAPK can be blocked with the flavone-derivative PD 98059 by inhibiting its phosphorylation. In light of these findings, it seems feasible to investigate in clinical studies if administration of an MAPK inhibitor reduces complications after major trauma and hemorrhagic shock. Data from our study indicate that inhibition of the p44/42 pathway results in an attenuation of inflammatory mediator release and decreased liver injury as evidenced by normal α-GST levels.

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ARCHIVES OF INTERNAL MEDICINE
From Prolonged Febrile Illness to Fever of Unknown Origin: The Challenge Continues
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Background: Epidemiological changes and the ongoing expansion of the diagnostic armamentarium warrant a regular update of the spectrum of diseases that present as prolonged febrile illnesses.

Methods: We prospectively collected a series of 290 immunocompetent patients referred to our university hospital between 1990 and 1999 with a febrile illness (temperature ≥38.3°C) of uncertain cause, lasting at least 3 weeks. Patients were categorized in 4 groups according to the timing of diagnosis: early diagnosis (within 3 in-hospital days or 3 outpatient visits), intermediate diagnosis (between days 4 and 7), late diagnosis (after day 7), and no diagnosis during index contact or follow-up.

Results: A final diagnosis was established early in 67 patients (23.1%), intermediate in 38 (13.1%), and late in 87 (30.0%). In the remaining 98 (33.8%), no diagnosis was made. The cause of the fever remained obscure in 50 (47.6%) of 105 patients with episodic fever vs 48 (25.9%) of 185 patients with continuous fever (P<.001). Among the 192 patients with a final diagnosis, noninfectious inflammatory diseases represented the most prevalent diagnostic category (35.4%), surpassing infections (20.7%), miscellaneous causes (19.8%), and malignancies (15.1%). Fourteen disorders accounted for over 59% of diagnoses, whether diagnosis was reached early, intermediate, or late. Hematological malignancies made up 11.5% of diagnoses, but were responsible for 14 (58.3%) of the 24 fatalities related to the febrile illness. Of the 80 patients discharged alive without diagnosis and for whom follow-up was available, 3 died, but the deaths were considered to be unrelated to the feverish illness.

Conclusions: Prolonged febrile illnesses remain a diagnostic challenge. Despite the technological progress of the late 20th century, the origin of the fever remains elusive in many patients, especially in those with episodic fevers. Noninfectious inflammatory diseases emerge as the most prevalent diagnostic category. (2003;163:1033-1041)

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