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Can we predict the effects of NF- κ B inhibition in sepsis? Studies with parthenolide and ethyl pyruvate

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Abstract

Background—Based partially on encouraging findings from preclinical models, interest has grown in therapeutic inhibition of NF- κ B to limit inflammatory injury during sepsis. However, NF- κ B also regulates protective responses, and predicting the net survival effects of such inhibition may be difficult.

Objectives—To highlight the caution necessary with this therapeutic approach, we review our investigations in a mouse sepsis model with parthenolide and ethyl pyruvate, two NF- κ B inhibitors proposed for clinical study.

Results—Consistent with published studies, parthenolide decreased NF- κ B binding activity and inflammatory cytokine release from lipopolysaccharide (LPS) stimulated RAW 264.7 cells *in vitro*. In LPS-challenged mice (C57BL/6J), however, while both agents decreased lung and kidney NF- κ B binding activity and plasma cytokines early (1 – 3 h), these measures were increased later (6 – 12 h) in patterns differing significantly over time. Furthermore, despite studying several doses of parthenolide (0.25 – 4.0 mg/kg) and ethyl pyruvate (0.1 – 100 mg/kg), each produced small but consistent decreases in survival which overall were significant ($p < 0.04$ for each agent).

Conclusion—While NF- κ B inhibitors hold promise for inflammatory conditions such as sepsis, caution is necessary. Clear understanding of the net effects of NF- κ B inhibitors on outcome will be necessary before such agents are used clinically.

Keywords

inflammation; NF- κ B; sepsis; therapy

1. Introduction

Even with broad-spectrum antibiotic treatment and intensive care unit (ICU) hemodynamic and pulmonary support, invasive infections causing sepsis and septic shock are associated

with a high mortality rate [1,2]. The incidence of sepsis is rising in the United States; this may be related to several factors, including increased use of chemotherapeutic and immunosuppressant agents and the advancing age of hospitalized patients. Excessive host inflammation plays a central role in the pathogenesis of the shock and organ injury occurring during sepsis [3]. Consequently, the development of adjunctive anti-inflammatory therapies to modulate key components in this response has been a goal for both researchers and clinicians over the past 30 years. However, the complexity and redundant nature of the inflammatory response, as well as its role in host defense and the compensatory mechanisms it triggers, makes this therapeutic approach a difficult one. Despite promising results from preclinical studies, an unsuccessful clinical experience, first with high-dose corticosteroids, and then with agents designed to selectively inhibit inflammatory mediators, such as TNF and IL-1, highlighted this difficulty [4,5]. Even recombinant human activated protein C, an antithrombotic agent with anti-inflammatory effects that appeared beneficial in septic patients in an initial trial, has had inconsistent effects in subsequent ones [6]. Other antithrombotic agents have also not been beneficial, and all have increased the risk of hemorrhage [7].

Despite this disappointing clinical experience, there is continued interest in defining the role of host mediators in the inflammatory response during sepsis, and developing agents to effectively modulate their activity. These efforts are increasingly being directed towards the intracellular signaling pathways and transcription factors controlling the host response during states of stress like sepsis [8,9]. NF- κ B is one such transcription factor that plays a critical role in a complex network of adaptive host responses, including ones to infection [10,11]. Despite the potential promise of targeting NF- κ B for sepsis, however, experience in our laboratory and others suggests that its effective modulation may prove as difficult as that of other host mediators previously targeted for the treatment of sepsis [12–14].

2. NF- κ B and sepsis

During localized infection like pneumonia, microbes and microbial products bind to host cell pattern recognition receptors (PRR) such as toll-like receptors 2 and 4 (TLR-2 and TLR-4) [15,16]. Once engaged, these PRR molecules trigger signaling pathways resulting in the activation of downstream gene transcription factors, of which NF- κ B is central [17–19]. These in turn regulate the expression of mediators critical to the host's inflammatory and defensive response. Cytokines, chemokines, nitric oxide and other molecules produced and released in this response stimulate local upregulation of adhesion molecules, creation of chemotactic gradients and vasodilation that together promote the extravascular recruitment and activation of neutrophils and lymphocytes necessary for early microbial clearance [10,20–26]. However, if these local defenses are overcome, microbes and microbial toxins can be released into the vascular space in sufficient concentrations to produce a systemic inflammatory response causing shock, organ injury and if severe enough, death.

NF- κ B consists of a family of subunit molecules including p50, p52, p65 (RelA), c-Rel and RelB [11,27]. These subunits can combine to form either homodimers or heterodimers. The combination with the highest concentration in many cells and one closely associated with the inflammatory events occurring during sepsis is the p50/p65 heterodimer. As originally described, this heterodimer typically resides in the cytoplasm in an inactive state, bound to a family of inhibitor molecules (I κ B) including I κ B- α , - β , - γ and - ϵ [27,28]. I κ B inactivates NF- κ B by binding to its nuclear localization signal (NLS) site. Upon cellular stimulation of PRR molecules by microbial products such as lipopolysaccharide (LPS) of Gram-negative bacteria and peptidoglycan of Gram-positive ones, signaling occurs that results in downstream I κ B protein phosphorylation by the I κ B kinase complex (IKK- α , - β and - γ in combination) [27,29]. After phosphorylation, I κ B is released and undergoes ubiquitination

and proteasomal degradation [30]. The p50/p65 heterodimer is then free to translocate to the nucleus, where it controls the expression of a wide range of genes critical to host defense and the inflammatory response [11].

Of note, there are two primary pathways via which NF- κ B signaling occurs: the classical (canonical) one, as outlined above, and an alternative one [12,27–30]. NF- κ B activation via the classical pathway is dependent on IKK β and IKK γ and the degradation of I κ B α . It is associated with translocation of the p50/p65 heterodimer, and is very important in the coordination of inflammatory and innate immune responses that would be highly relevant during early sepsis. The alternative pathway employs IKK α homodimers and NF- κ B-inducing kinase (NIK). It is associated with nuclear translocation of the p52/RelB heterodimer and controls B-cell maturation and lymphocyte organ formation [27,31]. These pathways are not entirely segregated, however, and it is likely that there are critical events occurring during the evolution of sepsis that are influenced by alternative as well as the classical signaling pathways [32].

In addition to turning on the production of molecules such as cytokines, chemokines and nitric oxide, NF- κ B can inhibit apoptosis and prolong inflammatory cell survival [33–35]. Furthermore, NF- κ B increases the expression of some molecules such as TNF and IL-1, which can amplify activation of NF- κ B via TNF and IL-1 receptor-mediated pathways [36]. Notably, while NF- κ B clearly has an important role in initiating a pro-inflammatory response, it also participates in the resolution of inflammation [37]. Finally, NF- κ B activates several feedback mechanisms that directly control the stimulatory effects of p50/p65. For example, other NF- κ B subunit combinations such as p50 homodimers may be activated and bind to κ B consensus sites in the nucleus to repress transcription. Also, the p50/p65 heterodimer itself stimulates production of I κ B, its cytosolic inhibitor.

Several lines of evidence have implicated NF- κ B in the pathogenesis of organ injury and lethality during sepsis. In *in vivo* models, both LPS and bacteria challenge have been shown to increase NF- κ B activation in a variety of different tissues [11,38,39]. More importantly, experiments in genetically engineered animals or with direct or indirect inhibitors of NF- κ B have demonstrated that this activation stimulates the production of the inflammatory mediators and patterns of myocardial, lung, hepatic, and renal injury associated with sepsis in humans [40–55]. Furthermore, in some – although not all – clinical studies testing it, activation of NF- κ B in differing cell types has been shown to correlate with the severity of sepsis and organ injury, as well as with lethality itself [56–59].

Importantly, though, while NF- κ B activation may contribute to inflammatory tissue injury during sepsis, it also has a critical role in host defense as well as other protective cellular responses [12,33,34,37,60,61]. Therefore the net effects of agents designed to inhibit NF- κ B must be evaluated in models where survival is an end point. Experience, both in animal models of sepsis and in septic patients, has shown that improvement in cardiopulmonary and other organ function following treatment with anti-inflammatory agents may not correlate with survival [62,63]. Moreover, many factors related to both the infectious challenge as well as the regimen of treatment itself may alter the effects of immunomodulators in sepsis [5]. In a recent review of published studies, while NF- κ B inhibition consistently improved survival in LPS-challenged sepsis models, it appeared less beneficial, and in some cases harmful, in bacteria-challenged models [11].

In order to further explore the potential applicability of therapeutic NF- κ B inhibition in sepsis and to investigate the variables that might alter the effectiveness of this approach, the survival effects of two agents, parthenolide and ethyl pyruvate, were investigated in our laboratory [13,14]. Both agents had been reported to inhibit NF- κ B, to be efficacious in

LPS- and bacteria-challenged sepsis models, and had been considered potential candidates for clinical investigation [64,65]. Initial experiments were designed to confirm the reported benefits of these two agents in LPS-challenged models alone prior to examining them further in bacteria models of sepsis. However, the findings from these studies with LPS differed from prior promising reports, and in combination they highlight how difficult it may be to predict the effects of this therapeutic approach.

3. Studies with parthenolide and ethyl pyruvate in an LPS-challenged mouse model

3.1 Parthenolide

Parthenolide is a sesquiterpene lactone derived from Mexican-Indian medicinal plants (feverfew, *Tanacetum parthenium*) [66]. It and related agents incorporate an exocyclic methylene group in conjugation with the carbonyl group of a γ -lactone that can inactivate sulfhydryl groups necessary for NF- κ B activity [66,67]. Some studies show that parthenolide and other sesquiterpene lactones can alkylate and inactivate a cysteine residue in the p65 subunit necessary for DNA binding [68,69]. In other studies, these agents were shown to alkylate a cysteine residue in IKK preventing the phosphorylation and degradation of I κ B- α [70–72]. In rats challenged with intraperitoneal LPS and observed for up to 5 h, parthenolide and a related sesquiterpene lactone, isohelenin, inhibited nitric oxide levels and improved hemodynamics [73,74]. Although NF- κ B levels appeared reduced with parthenolide in published studies, these were measured only for 2 h, and inflammatory cytokine levels and survival were not reported. In accompanying studies in mice, some but not all doses of these two agents increased survival with LPS challenge. In rats undergoing cecal ligation and puncture, parthenolide reduced NF- κ B levels for up to 6 h and TNF, IL-6 and IL-10 at 18 h, but later mortality was not altered significantly [75]. Based on these studies, the effects of parthenolide were evaluated in our laboratory, first *in vitro* and then in mice challenged with intraperitoneal LPS [13].

In RAW 264.7 cells, exposure to increasing LPS (*E. coli* 0111:B4) concentrations produced dose-dependent increases in NF- κ B binding activity measured at 1 h (*Trans*-AM ELISA, Active Motif, Carlsbad, CA) (Figure 1A). In cells exposed to a single concentration of LPS, treatment with increasing doses of parthenolide produced dose dependent decreases in NF- κ B binding activity (Figure 1B). Finally, when examined over time from 0.25 to 9 h, LPS challenge caused early increases in NF- κ B binding activity that then decreased over time and were similar to unstimulated control cells by 3 h (Figure 1C). Compared to LPS alone, cells treated with parthenolide had decreases in NF- κ B binding activity and were similar to unstimulated cells by 0.5 h. LPS stimulation also caused increases in the levels of 13 cytokines measured (including IL-1 β , IL-6, IL-10, and TNF- α), but different from NF- κ B, these increases became progressively greater over the 9-h observation period. Parthenolide treatment reduced these cytokines, and these decreases were greater at later time-points.

Experiments then assessed the effects of intraperitoneal (IP) LPS challenge and parthenolide treatment on lung and kidney NF- κ B binding activity (and an NF- κ B index reflecting the combined changes in these organs) and on plasma cytokine levels in C57/BL6J mice. Compared with saline challenge, nonlethal LPS (2.5 mg/kg, IP) increased NF- κ B binding activity early (1 – 3 h) and late (6 – 9 h) (all $p < 0.05$ for each parameter over each period) (Figure 2A). Compared with nonlethal LPS, lethal LPS (40 mg/kg, IP) was associated with further increases in NF- κ B binding activity at both time-points, but these were only significant or approached significance late (Figure 2B). In animals challenged with lethal LPS, compared with placebo, parthenolide treatment (1.0 mg/kg, IP, immediately following LPS) was associated with reductions in lung and kidney NF- κ B binding activity and in the

NF- κ B index early, but with increases late, although these changes were not significant (Figure 2C). However, the early decrease and later increase in NF- κ B index with parthenolide occurred in patterns that were significantly different ($p = 0.02$ for the differing effect of parthenolide comparing early versus late).

Compared with saline challenge, nonlethal LPS was associated with significant increases in 12 of 13 cytokines both early and late (Figure 2D). Compared with nonlethal LPS, lethal LPS was associated with further increases in cytokines at both time periods but these reached significance primarily late (Figure 2E). In animals challenged with lethal LPS, compared with placebo, parthenolide was associated with reductions in 11 cytokines early and increases in 11 of these late (Figure 2F). Although none of these effects were individually significant, their overall patterns of change differed significantly comparing early and late time-points ($p = 0.001$).

In survival studies, challenge with intraperitoneal LPS (40 mg/kg) in mice treated with diluent (placebo) resulted in lethality rates of 80%. Different from previous reports, however, compared with placebo each of five differing doses of parthenolide (0.25 – 4 mg/kg, IP) administered immediately following LPS reduced survival and increased the hazard ratio of death (Cox proportional hazards model) (Figure 3A). While none of these decreases in survival with any one dose were significant, they were all very consistent and overall, parthenolide significantly worsened the hazard ratio of death ($p = 0.04$) (Figure 3A and 3B). Parthenolide administered to normal animals did not alter survival.

Thus, *in vitro*, parthenolide clearly decreased NF- κ B binding activity and inflammatory cytokine expression. However, early decreases in these parameters with parthenolide in LPS-challenged mice were followed by later increases, and the overall effect of parthenolide on survival was a harmful one. To further test the potential effects of NF- κ B inhibition in this model, we investigated treatment with ethyl pyruvate [14].

3.2 Ethyl pyruvate

Pyruvate is a product of cytosolic glycolysis and a precursor for mitochondrial intermediary metabolism. As an α -keto carboxylate, it can also act as an endogenous antioxidant that scavenges reactive oxygen species (ROS) [76–80]. Based on this latter property, exogenous pyruvate was considered to be a potential antioxidant therapeutic. To improve its solubility, one group of investigators formulated ethyl pyruvate (EP), a derivative of pyruvic acid in a balanced salt solution [81]. In non-septic models, EP improved survival and other outcome measures in intestinal ischemia–reperfusion, hemorrhagic shock, and alcohol- and cholestasis-induced injury models [81–86]. In sepsis models, EP limited tissue injury and improved survival with either LPS or bacteria [87–90]. Of note, EP not only reduced oxidant injury in these models, but also inhibited NF- κ B (p65) and downstream inflammatory mediators such as TNF- α , interleukin-6 (IL-6) and nitric oxide (NO). Studies *in vitro* suggested that ethyl pyruvate inhibited p65 by covalently modifying cysteine residues necessary for its DNA binding [91]. Based on the results of these *in vitro* and *in vivo* studies, patents were obtained for clinical application of ethyl pyruvate for ischemia–reperfusion injury and cytokine-mediated inflammatory conditions such as sepsis [92,93]. A Phase II trial was performed with EP in patients requiring cardiopulmonary bypass [94]. Despite promising results with ischemia and reperfusion in animal studies, EP was not beneficial in this trial.

Based on the effects reported with EP in *in vivo* sepsis models, this agent was also investigated in our mouse model [14]. However, findings from these studies were very similar to prior ones with parthenolide. Compared to saline-challenged animals, LPS (30 mg/kg, IP) increased NF- κ B binding activity significantly in both lung and kidney at 3 h and

in lung at 9 h ($p < 0.0001$ for both) (Figure 4A). In both organs, NF- κ B binding activity was greater at 3 versus 9 h ($p = 0.002$ for each). When given immediately following LPS, compared with placebo, EP (100 mg/kg, IP) had significantly different effects on lung NF- κ B binding activity at 3 versus 9 h ($p = 0.05$ for the differing effect of EP at 3 vs 9 h) (Figure 4B). While EP decreased lung NF- κ B binding activity significantly ($p = 0.05$) at 3 h, treatment increased it at 9 h, although not significantly. In a similar pattern, EP was also associated with an early decrease and later increase in kidney NF- κ B binding activity, but these changes were not significant.

Compared with saline challenge, LPS significantly increased almost all cytokines at both 3 and 9 h ($p = 0.05$ for each time-point) (Figure 4C). Compared with placebo, EP did not alter any individual cytokine significantly at either time-point (Figure 4D). However, as with lung NF- κ B levels, EP was associated with early decreases and later increases in IL-1 β , IL-2, IL-10, TNF- α and JE that were significantly different comparing the two time-points ($p = 0.05$ for both). Overall in LPS animals, EP was associated with decreases in 11 of 12 cytokines at 3 h and increases in 11 of 12 cytokines at 9 h in patterns that were very different comparing the two time-points ($p < 0.0001$ for the differing effects of EP comparing 3 vs 9 h). Finally, LPS increased NO levels at 3 and 9 h, and these changes were greater at the latter time ($p = 0.006$ for all comparisons) (Figure 4E). In a pattern that was not significant but was similar to both NF- κ B and cytokine levels, EP was associated with a decrease in NO early and an increase later (Figure 4F).

Most notably, however, similar to the effects of parthenolide, with lethal LPS challenge (30 mg/kg, IP) each of six different doses of EP tested (0.01 – 100 mg/kg) administered immediately after LPS worsened survival and increased the hazard ratio of death (Cox proportional hazards model) (Figure 5A). Although none of these changes were individually significant, they were all highly consistent. When combined, the overall effect of EP on increasing the hazard ratio of death was significant ($p = 0.01$) (Figure 5A and 5B).

4. Conclusion

Considerable data indicates that activation of NF- κ B contributes to the systemic inflammatory response and its pathogenic effects during sepsis [11]. However, NF- κ B also has adaptive and protective roles during this response [12,36,37,60,61]. Thus, inhibiting NF- κ B might very well have unpredictable effects on survival during sepsis.

Parthenolide and EP, two potential NF- κ B inhibitors, have both shown beneficial effects in preclinical models of sepsis related to both noninfectious and infectious challenges. In the studies conducted in our laboratory, however, these two agents produced unexpected and concerning findings when considered in the context of these prior investigations. Consistent with their reported effects, both agents inhibited early (3 h) increases in NF- κ B binding activity and inflammatory cytokines related to LPS challenge. After this inhibition, however, both agents were also associated with later (9 h) increases in these same mediators in patterns that were significantly different comparing time-points. Furthermore, despite studying a range of doses, neither agent improved survival following LPS challenge, and when the effects of each were combined across the doses studied, both worsened survival significantly.

Although not previously reported, the divergent effects of parthenolide and EP on NF- κ B binding activity and inflammatory cytokines across time may relate to several factors acting alone or in combination. NF- κ B activation has been reported to occur in a biphasic pattern after LPS stimulation in mice, with the first peak occurring at 0.5 – 2 h and the second at 8 – 12 h [35]. The first peak appears related to a direct stimulatory effect of LPS, and the second to a subsequent rise in TNF- α and IL-1 β levels. While the regimens of parthenolide and EP

studied may have been sufficient to inhibit the first peak of activity, this may not have been the case for the second one. In addition, early inhibition of anti-inflammatory cytokines such as IL-10 may have resulted in larger increases in TNF- α and IL-1 β later, which in turn caused a rebound in NF- κ B. Independent of whether these or other mechanisms were at work, prior review of the *in vivo* experience testing the effects of these agents with LPS showed that analysis of their effects on NF- κ B over time was very limited [13,14]. While prolonging treatment with either agent may have prevented the later increases in NF- κ B noted, understanding the influence of time on such effects appears important.

The divergent effects of parthenolide and EP on NF- κ B and inflammatory cytokine levels provide one basis for the worsened outcome we noted. The contrasting effect these treatments had on survival when compared with studies by other laboratories may relate to other factors as well – such as differences in the strains or numbers of mice studied, the lethality of LPS challenge, and the doses of treatment investigated [13,14]. We studied a range of doses of each agent in relatively large numbers of animals. Furthermore, we employed LPS challenges that were not entirely lethal, to permit detection of either beneficial or harmful treatment effects. Consistent with our findings, LPS challenge in transgenic mice unable to activate endothelial NF- κ B results in increased endothelial cell apoptosis, vascular permeability and mortality compared to wild-type mice [95]. Also, mice deficient in NF- κ B subunits p50 and p65 were more sensitive to the lethal effects of LPS challenge [96]. Finally, consistent with the adverse effects of EP in mice, in a nonlethal LPS-challenged porcine model, EP was not associated with any beneficial physiologic effect, but was reported to significantly worsen anion gap acidosis [97].

Other factors, however, may also have contributed to the lack of benefit noted with parthenolide and EP in our mouse model as compared to other studies. Additional dosing or continuous infusion with the agents may have proven beneficial. Also, endotoxemia may not simulate key pathogenic events in sepsis, and treatment in a bacteria model may have shown benefit. However, a literature search and meta-analysis our group conducted of published studies assessing the effects of EP on survival in sepsis models did not find a variable to consistently explain why this therapy appeared beneficial in some experiments but not others [14]. As additional studies become available testing these agents, it will be useful to continue to consider this experience together in an attempt to define potentially influential variables [98]. It is also possible, however, that parthenolide and EP were not sufficiently specific inhibitors of NF- κ B to show benefit in our mouse model. Use of more selective NF- κ B inhibitors or genetically engineered animals may have demonstrated different effects and better supported the potential usefulness of this therapeutic approach.

It should be noted that parthenolide and EP have actions other than inhibition of NF- κ B that may have added to their effects in our studies. Both agents have been reported to have antioxidant activity [73,76–80,99]. While this activity may contribute to NF- κ B inhibition, it could have additional anti-inflammatory and other effects as well. Also, EP has been shown to exert anti-inflammatory actions via mechanisms such as inhibition of glycoxalases (GLO) and STAT3-dependent signaling [100,101].

While our studies focused on the effects of parthenolide and EP on production of the pro-inflammatory cytokines that are commonly associated with the pathogenesis of sepsis, measuring changes in apoptosis with treatment may have also been informative [102]. Apoptosis has been associated with the pathogenesis of sepsis, and its inhibition has been shown to improve outcome in several different preclinical models [103]. Inhibition of NF- κ B has variable effects on apoptosis, dependent in part on cell type [102]. It is therefore possible that the outcomes noted with parthenolide and EP in our mouse studies were related to adverse effects on apoptosis.

In summary, both parthenolide and EP can inhibit NF- κ B and the inflammatory mediators this transcription factor regulates. While these agents have had promising effects in some preclinical models testing them, in others they have either not been beneficial or, as in ours, have had harmful effects. Importantly, in terms of published preclinical reports, our findings regarding the potential harmful effects of parthenolide and EP in sepsis models are in a minority. More published reports have suggested benefit than harm with these agents. These positive studies clearly support the continued investigation of these or related agents for the treatment of sepsis and other systemic inflammatory states. However, based on NF- κ B's complex role in regulating both maladaptive and protective host responses, such a combined experience emphasizes the caution necessary as potential inhibitory agents of this critical transcriptional factor are considered for clinical use.

5. Expert opinion

There are several steps in the activation, nuclear translocation, DNA binding, and transactivation of NF- κ B that can be targeted to inhibit this all-important transcriptional factor [104]. The list of agents that have been demonstrated to provide such inhibition is extensive and continues to grow [104]. While the number of these agents that have been studied preclinically for specific use in sepsis is much smaller, this list is also likely to grow [11]. Ultimately, there may be sufficient *in vitro* and preclinical *in vivo* evidence to support clinical application of such agents for patients with, or at risk of developing, infection and sepsis.

However, patients with sepsis and septic shock are complex and fragile. In considering the future application of agents designed to inhibit a host mediator with functions as complex as NF- κ B, it is worth further considering our past experience with other anti-inflammatory agents in sepsis. During the 1980s and 1990s, there was great interest in the use first of high-dose glucocorticoids and, later, mediatorspecific anti-inflammatory agents (e.g., TNF-directed mAbs, TNF-soluble receptor, and IL-1 receptor antagonist) [4,5]. Of note, among their recognized actions, all these agents had the potential to inhibit NF- κ B, and all had been reported to improve survival significantly in preclinical sepsis models. However, none of these agents showed significant benefit in repeated clinical trials, and high-dose corticosteroids may have been harmful. Even rhAPC, an antithrombotic and anti-inflammatory agent that was beneficial in preclinical sepsis studies and which contributed to a patent controversy regarding therapeutic NF- κ B inhibition, has shown inconsistent benefit in clinical sepsis trials, while consistently increasing the risk of hemorrhage [6,105–110].

Overall, therefore, many anti-inflammatory agents with promising effects in preclinical sepsis models failed in clinical trials, and in some cases may have been harmful. Unlike animal models, patients presenting with sepsis are a highly diverse group with respect to the underlying cause of sepsis (e.g., type, site, severity and duration) as well as the presence of other comorbid conditions [1,2]. It has become increasingly evident that such variables, which are typically not examined in preclinical models, can influence the net effects of immunomodulators in sepsis [5,6,111–116]. Furthermore, while excessive inflammation may be harmful, an integrated inflammatory response is also essential for host defense – so anti-inflammatory agents, while beneficial under one set of circumstances, may be harmful under another. Analysis of the preclinical and clinical experience with mediator-specific anti-inflammatory agents suggested that the severity of sepsis and the risk of death associated with it may have been important variables influencing these therapies [5]. These same variables may have altered the effects of rhAPC in septic patients; the FDA accounted for this when it restricted use of this drug to patients with a high risk of death [6]. Other studies have also suggested that site and type of infection can alter the effects of immunomodulators in sepsis [113–116]. Moreover, sepsis in animal models is typically

fairly brief, since animals succumb or recover quickly. This differs from patients with sepsis, who are often provided with aggressive cardiopulmonary support in addition to measures to eradicate infection. Thus, the influence of duration of sepsis on the effects of immunomodulators is much more difficult to investigate in animal models. However, this may be a critical variable when considering the applicability of an anti-inflammatory agent for a septic patient. While use of such an agent might be most beneficial early, when pro-inflammatory mediator production is pronounced, it may be harmful in the later stages, when the host's adaptive anti-inflammatory response has already been activated.

Our studies with parthenolide and EP were done with LPS, a noninfectious challenge that may be less susceptible to some variables potentially influencing anti-inflammatory agents during live bacterial infection in patients. Despite this, our results differed from those of other groups studying these agents with similar types of challenges. While these differences may be due to several factors (e.g., regimen of treatment, mouse strain, severity of challenge), they highlight the importance of confirmative results from several laboratories before the application of such agents clinically.

Although the overall experience with anti-inflammatory agents in the treatment of sepsis has been a disappointing one to date, it has also been highly instructive. It has become increasingly clear that the heterogeneity evidenced in septic patients must be accounted for during the investigation of new agents, at both preclinical and clinical stages. While therapies designed to inhibit NF- κ B may show great promise for the treatment of sepsis, their investigation before clinical use must be comprehensive. Furthermore, if clinical trials testing such agents occur, they will have to be monitored carefully to identify subgroups at potential risk with treatment.

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Figure 1. In *in vitro* studies in macrophage RAW 264.7 cells following 1-h incubations, increasing LPS doses produced graded increases ($p = 0.01$) in NF- κ B (Panel A) and increasing doses of parthenolide with LPS stimulation produced graded reductions ($p < 0.0001$) in NF- κ B (Panel B) In incubation studies performed from 0.25 – 9 h, LPS increased NF- κ B up to 1 h ($p < 0.0001$) and these increases were inhibited with parthenolide ($p = 0.002$) (Panel C). For these studies, nuclear protein was extracted from cell pellets, the protein concentration determined and NF- κ B DNA binding activity assays were performed using a *Trans-AM* ELISA-based kit from Active Motif (Carlsbad, CA). This assay employed an oligonucleotide containing the NF- κ B p65 consensus binding site and an antibody specific for p65, to isolate and detect this subunit respectively [13].

Figure 2. In studies measuring the effect of LPS and parthenolide on tissue expression of NF-κB (Panels A, B and C) and plasma cytokine levels (Panels D, E, and F), C57BL/6J mice (n = 241) were challenged with saline, nonlethal dose LPS (2.5 mg/kg), or lethal dose LPS (40 mg/kg)[13]. Lethal-dose LPS animals were also randomized to parthenolide (1.0 mg/kg) treatment or diluent. Early (1 or 3 h) or late (6, 9 or 12 h) animals were randomly selected for plasma cytokine and lung and kidney NF-κB measurement. Compared with saline, nonlethal LPS increased lung and kidney NF-κB and the NF-κB index (based on measures obtained in both organs) both early and late (Panel A). Compared to nonlethal LPS, lethal LPS increased lung and kidney NF-κB and the NF-κB index early and late, but these were only significant (or approaching it) late (Panel B). With lethal LPS, compared with placebo, parthenolide decreased these measures early and increased them late, and this difference over time was significant for the NF-κB index (Panel C). Compared with saline, nonlethal LPS increased cytokines significantly both early and late (except for IL-4) (Panel D). Compared with nonlethal LPS, lethal LPS was associated with increases in each cytokine both early and late (except for JE early), but only two of these were significant early, whereas 12 of them were significant late (Panel E). With lethal LPS, compared with placebo, parthenolide decreased 10 of the 13 cytokines early and increased 11 of 13 late, although none significantly (Panel F). However, the overall pattern of change in the proportion of cytokines – decreased early and increased late – with parthenolide was highly significant (p = 0.001) [13].

*p < 0.05. **p = 0.08.

Figure 3. To test the effects of parthenolide on survival, C57BL/6J mice challenged intraperitoneally (IP) with LPS (40 mg/kg) (*E. coli* 0111:B4, Sigma, St. Louis, MO) were randomized to receive similar volumes of either diluent (control) or one of five doses of parthenolide (0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg)

Animals alive at 168 h were considered survivors. Each dose of parthenolide resulted in reduced survival compared with control and an increase in the hazard ratio of death (Panel A), although none significantly. However, the overall increase in the hazard ratio with parthenolide was significant ($p = 0.04$) (Panels A and B) [13].

*Significance level for the effect of parthenolide on the hazards ratio.

Figure 4. In studies measuring the effect of LPS and ethyl pyruvate on tissue expression of NF- κ B (Panels A and B), plasma cytokine levels (Panels C and D) and nitric oxide levels (Panels E and F), C57BL/6J mice (n = 108) were challenged with saline or with LPS (30 mg/kg) [14]. LPS animals were also randomized to ethyl pyruvate (100 mg/kg) treatment or diluent. Early (3 h) or late (9 h) animals were randomly selected for plasma cytokine and nitric oxide levels followed by lung and kidney NF- κ B measurement. Compared with saline challenge, LPS increased NF- κ B levels significantly in both lung and kidney at 3 h, and in lung at 9 h (Panel A). In both organs, changes with LPS were greater early compared to late. LPS, compared with placebo ethyl pyruvate, had significantly different effects on lung NF- κ B levels at early versus late (Panel B). While ethyl pyruvate decreased lung NF- κ B early, it increased NF- κ B late. In a similar pattern, ethyl pyruvate was also associated with an early decrease and later increase in kidney NF- κ B levels, but these changes were not significant. Compared with saline, LPS increased almost all cytokines significantly both early and late (Panel C). With LPS, compared with placebo, ethyl pyruvate did not alter any individual cytokine significantly at either time-point (Panel D). However, as with lung NF- κ B levels, ethyl pyruvate was associated with early decreases and later increases in IL-1 β , IL-2, IL-10, TNF- α and JE that were significantly different comparing the two time-points. Overall, ethyl pyruvate was associated with decreases in 11 cytokines early and increases in 11 late in significantly different patterns. Finally, LPS increased NO levels early and late, and these changes were greater later (Panel E). In a pattern that was not significant, but was similar to both NF- κ B and cytokine levels, ethyl pyruvate was associated with a decrease in NO early and an increase later (Panel F) [14].

*p < 0.05 for the effect of LPS.

†p < 0.05 comparing the effect of EP at 3 versus 9 h for the individual cytokine.

Figure 5. The survival effects of six different doses of ethyl pyruvate (0.01, 0.1, 1, 10, 50, and 100 mg/kg) were studied as parthenolide had been studied in C57BL/6J mice [14]

Each dose of ethyl pyruvate resulted in a survival rate that was reduced compared with control and increased the hazard ratio of death (Panel A). The overall increase in the hazard ratio with ethyl pyruvate was significant ($p = 0.01$) (Panels A and B) [14].

*Significance level for the ethyl pyruvate on the hazards ratio.