Prefatory Note – A Regimen for Ebola

Several weeks ago I wrote a manuscript positing that certain readily available nutraceuticals and drugs have potential for decreasing mortality in septic shock, reasoning from findings in cell culture and rodent studies; these agents included: spirulina, lipoic acid, glycine, high-dose folate, and metformin or berberine.

Subsequently, encountering the literature on Ebola, I was surprised to learn that, while sepsis is triggered by gram negative bacteria and Ebola by an RNA virus, the pathogenic mechanisms whereby they kill patients are substantially homologous, involving heavy release of pro-inflammatory cytokines from macrophages, circulatory shock, vascular hyperpermeability with edema, and disseminated intravascular coagulation. This may be explained by the recent observation that the membrane glycoprotein of Ebola activates macrophages/monocytes via toll 4 receptors, just as does endotoxin. I therefore concluded that the measures I had recommended for treatment of sepsis in all likelihood would be beneficial for treatment of Ebola – not for remediating the infection, but for preventing the downstream consequences of the infection that often prove to be fatal.

Moreover, in light of a recent report that induction of the enzyme heme oxygenase-1 slows the proliferation of Ebola virus within cells, the heme oxygenase inducer lipoic acid may have clinical potential for slowing the spread of Ebola within the body, possibly giving the immune response a better chance to cope with it.

Although my recommendations are rooted in logical analysis rather than clinical experience, and hence must be considered hypothetical, they are likely to be quite safe and reasonably practical, and, in light of the Ebola crisis currently afflicting Africa, for which clinically proven strategies are not yet available, it would not seem unreasonable to try them clinically on a pilot basis at the current time. Indeed, my friend and colleague Dr. Jeremy Stone has urged that these strategies should be given serious consideration now.

The dose schedules which I would judge likely to have some efficacy – and to be safe – for management of both sepsis and Ebola infection are:

Spirulina – 15-30 g daily, orally (or phycocyanin – 2-4 g daily)

Glycine – 5 g, 4 times daily, orally

Folate – 40 mg, twice daily, orally

Lipoic Acid – 600 mg, three times daily

Metformin – 850 mg, twice daily, orally; or Berberine - 500 mg three times daily, orally

Biliverdin/Phycocyanobilin, Lipoic Acid, Glycine, High-Dose Folate, and Metformin/Berberine as Antidotes to Endotoxin

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Abstract

Endotoxin's activation of the TLR4 receptor mediates many of the life-threatening complications of gram-negative septicemia. TLR4 signaling includes an arm in which ASK1-mediated activation of p38 MAP kinase promotes transcription, and/or prolongs the mRNA half-life of inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and pro-inflammatory cytokines. TLR4-mediated activation of ASK1 is contingent on generation of reactive oxidant species via NADPH oxidase activation; this dissociates ASK1 from its inhibitor thioredoxin, enabling it to interact with TRAF6 in the TLR4 signaling platform. Activation of NADPH oxidase also plays a poorly defined role in up-regulating TLR4 activation of NF-kappaB. Hence, inhibitors of NADPH oxidase importantly down-regulate TLR4 signaling, while also lessening the oxidative stress and peroxynitrite formation associated with inflammation. Bilirubin functions physiologically to inhibit certain NADPH oxidase complexes, and administration of bilirubin and its immediate precursor biliverdin confers protection in rodent models of gram-negative sepsis. The biliverdin homolog phycocyanobilin (PhyCB), richly supplied by cyanobacteria such as spirulina, appears to mimic the inhibitory impact of bilirubin on NADPH oxidase activity; this likely explains why administration of phycocyanin, the cyanobacterial protein which contains PhyCB as a chromophore, likewise provides protection from endotoxin in rodent models. Intravenous or oral administration of PhyCB or of biliverdin may have clinical potential for reducing morbidity and improving survival in gram-negative sepsis. Intracellular production of biliverdin in macrophages and other tissues can be promoted by the phase 2 inducer lipoic acid (LA), which boosts expression of heme oxygenase-1 and also stimulates glutathione synthesis; LA administration exerts marked anti-inflammatory activity and aids survival in rodent models of sepsis. High intakes of the amino acid glycine may likewise have potential in this regard. An influx of calcium through voltage-sensitive L-type calcium channels is a key mediator of LPS-induced NADPH oxidase activation, and of other LPS-stimulated signaling pathways that boost macrophage production of pro-inflammatory cytokines and prostanoids. In macrophages, glycine opposes LPS-triggered calcium influx by activating chloride channels that hyperpolarize the plasma membrane, thereby opposing opening of L-type calcium channels; hence, in vitro, glycine suppresses the ability of LPS to induce oxidative stress and increase TNF-alpha expression in macrophages. Moreover, glycine-enriched diets have been reported to enhance survival in mice injected with LPS, and to lessen the inflammation and tissue injury in the liver and lungs of such animals. And there are recent reports that supraphysiological doses or concentrations of folic acid lessen the pro-inflammatory impact of LPS, likely because the reduced folate metabolites which prevail intracellularly have versatile oxidant scavenging activity, including the ability to scavenge peroxynitrite-derived radicals. AMPK activators such as metformin and berberine, by intervening in the signaling pathway that boosts tissue factor expression in macrophages/monocytes, may lessen risk for disseminated intravascular coagulation. Biliverdin/PhyCB, high-dose folate, and metformin/berberine all have potential to act directly on endothelial cells to lessen the vascular hyperpermeability associated with sepsis. Since Ebola infection triggers a sepsis-like syndrome by activating TLR4 in macrophages and dendritic cells, these measures may also be useful in this disorder. Moreover, heme oxygenase induction

slows the intracellular proliferation of the Ebola virus; hence, LA and possibly biliverdin and PhyCB may have clinical potential for slowing the spread of Ebola within the body.

TLR4 Signaling in Sepsis

Endotoxin (lipopolysaccharide – LPS) is a key mediator of the organ failure and mortality associated with severe gram-negative infections, most notably in the elderly, and also, via activation of Kupffer cells, plays a cofactor role in alcoholic liver disease and other hepatic disorders associated with leaky gut syndrome, including possibly non-alcoholic fatty liver disease.[1-5] LPS activates the TLR4 receptor, and thereby promotes expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (cox-2), tissue factor (TF), and a range of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in macrophages. In other tissues, such as vascular endothelium, concurrent interferon-γ activity is required for induction of iNOS and cox-2.

LPS-mediated induction of iNOS is a key driver of the complications of sepsis; excessive production of NO induces hypotension, and the highly reactive peroxynitrite resulting from the spontaneous interaction of NO and superoxide is a mediator of the organ failure associated with sepsis. The iNOS promoter has binding sites for AP-1, NF-kappaB, STAT1 homodimer, and interferon response factor 1 (IRF1).[6-9] LPS activity induces binding of AP-1 and NF-kappaB to this promoter, whereas interferon-γ induces binding of the latter two. The activated IFNγ receptor stimulates tyrosine phosphorylation of Jak2 and Stat1; the latter, as a homodimer, induces transcription of IRF1, which can then bind to the iNOS promoter.[10]

Binding of LPS to TLR4 triggers the assembly of a complex signaling platform capable of activating the MAP kinases p38, JNK, ERK1/2, and also IKKbeta; the latter promotes the proteasomal degradation of IkappaB and the translocation of NF-kappaB to the nucleus.[11] In aggregate, these effects induce the binding of AP-1 and NF-kappaB to the iNOS promoter in transcriptionally active forms. In mouse splenocytes, TLR4's activation of p38 MAP kinase is essential for optimal induction of iNOS, and requires complex formation between apoptosis signal-regulating kinase 1 (ASK1) and TRAF6, a component of the TLR4 signaling platform.[12] ASK1 is capable of activating both p38 and JNK MAP kinases.[13] Splenocytes derived from ASK1-knockout mice show a notable blunting of LPS-induced iNOS expression. LPS's capacity to induce the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-12 is also depressed in the absence of ASK1. Drug inhibitors of p38 have an impact comparable to ASK1 knockout on expression of iNOS and these cytokines; the up-regulatory impact of p38 activity on iNOS, cox-2, and cytokine expression reflects, at least in part, stabilization of their mRNAs.[14-20] The 3' untranslated regions of the mRNAs of many proteins that drive inflammation possess AU-rich elements capable of binding certain proteins which render these mRNAs susceptible to rapid degradation; activation of p38 and its downstream target MAP kinase-activated protein kinase-2 (MAPkap-2) reverses the impact of these proteins, either by direct phosphorylation, or by inducing the cytoplasmic localization of HuR, a protein which binds to AU-rich elements in a way that promotes mRNA stability.[14, 19, 21-24] Hence, p38 boosts the expression of an entire range of pro-inflammatory factors by stabilizing their mRNAs. ASK1 knockout does not impede LPS-mediated activation of JNK or NF-kappaB in macrophages; the key role of ASK1 in LPS-induced signal transduction appears to be activation of p38 MAP kinase. In LPS-stimulated macrophages and glial cells, p38 activity appears to be required for

increased nuclear expression of c-Jun and c-Fos, and hence of assembly of AP-1 on the iNOS promoter; curiously, c-Fos is one of those proteins whose mRNAs are stabilized by p38.[14, 25-27]

Oxidative Stress is Required for ASK1 Activation

Matsuzawa and colleagues have demonstrated that complex formation between ASK1 and TRAF6 in LPS-exposed splenocytes is contingent on concurrent LPS-induced oxidative stress; hence, co-incubation with the antioxidant N-acetylcysteine blocks LPS-triggered activation of ASK1 and p38.[12] TLR4-induced activation of NADPH complexes mediates this oxidative stress; in macrophages, TLR4 activates Nox4, whereas Nox2 can also be activated in vascular endothelial cells.[28-31] In macrophages, the signaling pathway which mediates this is complex, and appears to involve an increase in intracellular free calcium, and activation of c-Src, PI3K, Vav, and Rac2.[28, 32, 33] Complexation between thioredoxin and ASK1 is known to suppress ASK1 activity and promote its proteasomal degradation;[34, 35] oxidation of cysteine groups in thioredoxin alters its configuration so that it no longer binds to ASK1, freeing the latter such that it becomes susceptible to activation by TRAF6 or other mediators. Agents which block TLR4-mediated activation of NADPH oxidase activity can hence be expected to compromise the TRAF6-ASK1-p38 arm of TLR4 signaling, thereby blunting induction of iNOS and inflammatory cytokines. In murine macrophages, a chemical agent derived from flying squirrel droppings (!) that impedes LPS induction of oxidative stress, concurrently blocks complexation of TRAF6 and ASK1, downstream activation of p38 and AP-1, and induction of iNOS, cox-2, and pro-inflammatory cytokines.[25]

In microvascular endothelial cells exposed to LPS and INFγ, the NADPH oxidase inhibitors apocynin and DPI, as well as p47 deficiency, suppress induction of iNOS.[31] In these circumstances, binding of AP-1 and of IRF1 – but not of NF-kappaB – to the iNOS promoter is impeded. The effect on IRF1 may reflect the fact that p38 can confer an activating phosphorylation (Ser727) on STAT1 homodimers; STAT1 is then better capable of inducing transcription of IRF1.[8] In endothelial cells, LPS-mediated activation of JNK is suppressed by antioxidants (possibly reflecting ASK1 inhibition), and this contributes importantly to the observed inhibition of AP-1 activation.[31] In human tracheal smooth muscle cells, induction of cox-2 and phospholipase A2 by LPS is mediated by cytoplasmic translocation of HuR triggered by activation of p38, JNK, and Erk1/2; concurrent inhibition of NAPDH oxidase blocks all of these effects.[36]

Reactive Oxygen Species Also Up-Regulate TLR4 Activation of NF-kappaB

A number of studies indicate that TLR4-mediated activation of NADPH oxidase in the microenvironment of the receptor also up-regulates the signaling pathway(s) by which TLR4 agonists promote the canonical IKKbeta-dependent stimulation of NF-kappaB activity.[37-43] TLR4's ability to activate NF-kappaB is independent of ASK1, and it is not yet clear what protein ROS directly target in promoting this activation. Activation of TLR4 leads to assembly of a MyD88/IRAK1/IRAK4/TRAF6 complex that, through complex mechanisms entailing ubiquitination of TRAF6, activates the kinase TAK1; this kinase in turn confers activating phosphorylations on IKKbeta.[44] Stimulation of the kinase activity of IRAK4 is essential to this signaling pathway;[45, 46] transgenic knock-in mice expressing IRAK4 lacking kinase activity are completely resistant to septic shock.[47] There is a report that scavenging antioxidants (N-acetylcysteine, alpha-tocopherol) suppress activation of IRAK4 in LPS-treated neutrophils.[37] The interleukin-1 receptor likewise signals to NF-kappaB via formation of a MyD88/IRAK1/IRAK4/TRAF6 complex; measures which blocked NADPH oxidase activation or scavenged ROS were shown to inhibit recruitment of TRAF6 to this complex.[48] Hence, LPS-stimulated ROS production may be essential for

efficient formation and function of the MyD88/IRAK1/IRAK4/TRAF6 signaling module required for IKKbeta activation.

Activated TLR4 also stimulates the PI3K/Akt pathway;[49] Akt-mediated activation of IKKalpha, in turn, can induce a phosphorylation of p65 that boosts the transcriptional activity of NF-kappaB.[50] There is evidence that LPS-stimulated ROS production up-regulates this activation of PI3K/Akt, and hence acts in an additional way to promote the full stimulation of NF-kappaB activity.[37, 40, 42] Clearly, there is reason to suspect that agents that inhibit NADPH oxidase activity, or that antagonize the downstream impact of the ROS this activity generates, will lessen the capacity of LPS to activate NF-kappaB, a key transcriptional mediator of inflammation.

Bilirubin Interferes with TLR4 Signaling by Blocking NAPDH Oxidase Activation

Intracellular free bilirubin, generated via induction of heme oxygenase, functions as a potent physiological inhibitor of certain NADPH oxidase complexes, including those dependent on Nox2 and Nox4.[51-57] Hence, bilirubin has the potential to interfere with LPS-induced signaling by blocking formation of TRAF6-ASK1 complexes. In fact, in rodents administered LPS, or exposed to endogenous endotoxin via the cecal ligation and puncture (CLP - often employed as a model of sepsis), administration of bilirubin and its more soluble precursor biliverdin (rapidly converted to bilirubin within cells by ubiquitously expressed biliverdin reductase) has been found to confer notable protection. In rats infused intravenously with LPS, intraperitoneal administration of bilirubin (30 mg/kg) 30 minutes prior to LPS infusion completely prevented subsequent mortality; 40% of the rats died when infused with LPS without concurrent bilirubin treatment.[58] As compared to LPS-treated controls, those rats who also received bilirubin showed a notably lower rise in serum nitrate (reflecting decreased NO production), TNF-α, and serum transaminases; histological hepatotoxicity was attenuated, and hepatic induction of iNOS was blunted. In a further study, rats were infused intravenously with LPS in a dose that led to 80% mortality; the same dose was associated with only 12.5% mortality in rats pretreated with biliverdin (35 mg/kg i.p. 15 hours and again 1 hour prior to LPS injection).[59] This study, which focused on lung pathology, found that biliverdin pre-treatment notably blunted LPS-triggered lung inflammation and leukocyte accumulation. Another rat study employing the CLP technique examined the negative impact of endotoxin exposure on gastrointestinal motility and inflammation of jejunal muscularis.[60] Repeated i.p. injection of biliverdin (5 mg/kg) prior to and following laparotomy substantially ameliorated gastrointestinal dysmotility, influx of leukocytes, and induction of IL-6 and MCP-1; however, induction of iNOS in the jejunal muscularis was not blocked by biliverdin in this study. And in Gunn rats, in whom unconjugated plasma bilirubin levels are markedly enhanced owing to a genetic deficit of hepatic bilirubin conjugating activity, the mortality, hypotension, and iNOS induction associated with LPS infusion is significantly reduced.[51]

These anti-inflammatory effects of biliverdin/bilirubin in rodent models of endotoxin exposure may not be mediated solely by inhibition of NADPH oxidase activity. Bilirubin or biliverdin have shown an inductive effect on the anti-inflammatory cytokine IL-10 in some studies, including two of those cited above.[59-62] Conceivably, this may reflect bilirubin's agonist activity for the aryl hydrocarbon receptor (AhR), which in some cellular contexts promotes IL-10 transcription.[63-71] In lymphocytes, AhR interacts with c-MAF on the IL-10 promoter to induce IL-10 transcription and generate Tr1 suppressor cells.[71] In addition, interaction between biliverdin and cell-surface-expressed biliverdin reductase is reported to

activate that signaling pathway that promotes transcription of IL-10 in macrophages.[62] Hence, anti-inflammatory effects of IL-10 may complement the antioxidant and anti-inflammatory effects of NADPH oxidase inhibition in countering LPS toxicity. Moreover, in mouse macrophages, biliverdin's interaction with biliverdin reductase may induce the latter to bind to the promoter of the TLR4 gene, inhibiting its transcription.[72] Decreased expression of TLR4 would evidently blunt the adverse impact of endotoxin. Hence, there may be multiple complementary mechanisms whereby biliverdin/bilirubin blunt the toxicity of LPS. It should not go unmentioned that inhibition of NADPH oxidase should not only decrease induction of iNOS, but could be expected to oppose peroxynitrite formation by lessening the production of both superoxide and NO.

Phycocyanobilin Can Mimic the Antioxidant/Anti-inflammatory Effects of Bilirubin

Phycocyanobilin (PhyCB), a light-absorbing chromophore which is a major component of cyanobacteria such as spirulina – constituting about 0.6% of the dry weight of spirulina - is a derivative of biliverdin that can be converted by biliverdin reductase to phycocyanorubin, a homolog of bilirubin. PhyCB shares the ability of bilirubin to inhibit NADPH oxidase complexes, and it is suspected that this reflects intracellular conversion of PhyCB to phycocyanorubin, which is likely the direct inhibitor.[73-75] Oral pre-administration of phycocyanin, the spirulina protein which contains PhyCB as a covalently-attached chromophore, was reported to blunt dose-dependently the increase in serum concentrations of nitrite and TNF-α evoked by i.p. injection of LPS in mice.[76] In LPS treated macrophages in vitro, concurrent exposure to phycocyanin suppressed the rise in iNOS expression and NO production; NF-kappaB activation was also diminished by the phycocyanin.[77] In rats subjected to acute inflammatory lung damage by intratracheal administration of LPS, subsequent i.p. injection of phycocyanin (50 mg/kg) significantly blunted the rise in NO production, inflammatory cytokines, leukocyte influx, and edema observed in the lungs of these animals.[78] In cultured microglial cells, exposure to LPS increased expression of iNOS, Cox-2, TNF-α, and IL-6; concurrent exposure to phycocynin suppressed each of these effects.[79] These observations suggest that orally administered phycocyanin, or orally or parenterally administered PhyCB, might have clinical utility in sepsis by mimicking the physiological antioxidant/anti-inflammatory effects of biliverdin/bilirubin. Relative to biliverdin, PhyCB has the advantage that a concentrated natural source of it is readily available.

Phase 2 Inducer Lipoic Acid Suppresses LPS-Mediated Inflammation

As noted, heme oxygenase gives rise to intracellular free biliverdin/bilirubin by cleaving heme. Transcription of the inducible form of heme oxygenase, HO-1, is promoted by the nrf2 transcription factor, the activity of which in turn is boosted by a range of chemicals known as phase 2 inducers; these agents block the binding of nrf2 to Keap1, which functions to retain nrf2 in the cytoplasm and promote its proteasomal degradation.[80-82] In addition to amplifying HO-1 expression, nrf2 promotes transcription of the rate-limiting enzyme for glutathione synthesis, glutamate cysteine ligase, as well as a range of antioxidant enzymes.[83, 84] The oxidative stress induced by LPS in macrophages and other tissues also promotes nrf2 activation; this acts as a check on inflammation.[85, 86] Not surprisingly, mice in which nrf2 expression is knocked out have increased mortality and inflammation when treated with LPS, in comparison to wild type mice.[87] Elevated activation of NADPH oxidase contributes importantly to this effect.[88]

Lipoic acid (LA) is a safe and orally administrable phase 2 inducer whose clinical utility in diabetic neuropathy likely reflects its activity in this regard in vivo.[89-92] A great many studies have reported that LA can down-regulate LPS-induced inflammation, either in cultured cells or in rodents; suppressed activation of NF-kappaB plays an important role in this regard.[93-112] The protective utility of LA in LPS-induced acute lung injury in rats is largely attributable to HO-1 induction, as concurrent administration of an HO-1 inhibitor abrogates much of LA's anti-inflammatory activity.[101] However, other studies suggest that increased glutathione synthesis likewise contributes to the protection afforded by LA in sepsis models.[88] Glutathione functions to blunt LPS-mediated activation of NADPH oxidase by suppressing activation of PKC.[88, 113, 114] It may also act downstream from NADPH oxidase by antagonizing the oxidative impact of hydrogen peroxide on signaling proteins.[115, 116] Administered either orally or parenterally, LA has been found to reduce or delay mortality in rodent models of septic shock; not surprisingly, pre-administration achieves the greatest benefit, but post-administration also provides some protection.[94, 103, 108, 111]

Clinically, LA has been found to be safe and well tolerated in daily oral doses as high as 2400 mg.[117] 600 mg given 2-3 times daily has been found to be clinically useful in diabetic neuropathy.[92] It would be appropriate to evaluate doses of this magnitude in clinical sepsis.

Glycine - An Antagonist of Calcium Signaling in LPS-Treated Macrophages

An increase in intracellular free calcium (Ca_i) stemming both from increased calcium influx and a release of calcium from internal stores, is a crucial upstream mediator of NADPH oxidase activation in LPS-stimulated macrophages.[32, 33, 118] Macrophages express L-type voltage-dependent calcium channels, and these appear to be responsible for the triggered influx of extracellular calcium.[118-122] The LPS-induced increase in Ca_i, in addition to its stimulatory impact on NAPDH oxidase, works in other ways through mediators such as Ca^{+2}/calmodulin-dependent protein kinase II (CaMKII) to promote production of inflammatory cytokines and induction of cox-2; hence, an inhibitor of this kinase enhanced the survival of mice injected with a lethal dose of LPS.[123, 124]

Not surprisingly, drugs which inhibit L-type calcium channels markedly down-regulate the inflammatory activation of LPS-exposed macrophages.[118, 120-122, 125] However, such a strategy would presumably be contraindicated clinically, as these agents could potentiate the vasodilation and hypotension associated with septic shock. Fortunately, an alternative and safer strategy for suppressing calcium influx in macrophages has been discovered by Thurman and colleagues. Macrophages express glycine-gated chloride channels which glycine activates with a K_i of under 100 µM.[126, 127] *In vivo*, this activation is enhanced at plasma glycine levels achievable with high-dose oral administration of glycine.[128] In macrophages, the activation of these channels causes an inrush of chloride that hyperpolarizes the cell membrane, thereby antagonizing the LPS-induced opening of voltage-sensitive L-type calcium channels.[126] Yet high-dose glycine does not induce hypotension, and is well tolerated clinically.[129] *In vitro*, glycine was found to dose-dependently suppress the LPS-triggered increase in Ca_i, oxidative stress, and TNF-alpha expression.[32] And, most pertinently, feeding a glycine enriched diet (5% glycine by weight) was found to be markedly protective in mice injected intravenously with LPS; a dose of LPS which killed half of the control animals, failed to kill the animals consuming a high-glycine diet.[128] Dietary glycine was also markedly protective to mice subjected to a sub-lethal dose of LPS and concurrent partial hepatic ischemia-reperfusion.[128] The plasma levels of glycine achieved with the high-

glycine diet – 1.7 mM – were six-fold higher than those in mice fed a control diet. Studies of related interest have shown that dietary glycine can decrease inflammation and tissue damage to the lungs and liver in mice injected with LPS.[130, 131] Intriguingly, the glycine responsiveness of Kupffer cells was lost after four consecutive weeks of glycine feeding – suggesting down-regulation chloride channel expression – whereas the protection afforded to the lungs of LPS-treated mice was sustained.[131] This suggests that, for supplemental glycine to be optimally effective for alleviating LPS toxicity, its use should be reserved for acute-care situations.

High-Dose Folate Impedes TLR4 Signaling

There are several reports that supraphysiological concentrations or doses of folic acid can suppress the pro-inflammatory signaling of LPS, in macrophages and in mice.[132-134] In macrophages, folate concentration-dependently inhibits LPS-mediated induction of iNOS, TNFalpha, IL-1beta, as well as activation of NF-kappaB.[132] In pregnant mice, administration of high-dose folate (3 or 15 mg/kg) to pregnant mice injected with LPS blunted the ability of LPS to induce fetal death, intrauterine growth restriction, and preterm delivery; folate administration also decreased LPS-mediated NF-kappaB activation in the placentas of these mice.[134]

It is not likely that these LPS-antagonistic effects of high-dose folate reflect folate's essential nutritional activities, which are optimized by doses/concentrations of folate far lower than those employed in these studies. Rather, supraphysiological concentrations of folate have been shown to exert potent antioxidant effects, stemming from the fact that folate is reduced intracellularly to tetrahydro- derivatives that have versatile oxidant scavenging activity; in particular, reduced folates scavenge peroxynitrite-derived radicals.[135-137] The level of these reduced folate derivatives in cells rises as extracellular folate levels are increased through and beyond the physiological plasma concentration of folate; hence, high-dose folate has an antioxidant impact not seen with modest nutrional doses of this agent. Previous studies have shown that high-dose folate, as opposed to nutritional doses of folate, can recouple eNOS in dysfunctional vascular endothelium (likely owing to prevention or reversal of peroxynitrite-mediated oxidation of eNOS's essential cofactor tetrahydrobiopterin), markedly limit the myocardial damage induced by ischemia-reperfusion in rats, prevent induction of nitroglycerin tolerance, and decrease oxidative damage to DNA in diabetics.[138-143] Decades ago, Oster used high-dose folate (40-80 mg daily) with alleged success in the treatment of angina and intermittent claudication.[144] It therefore seems reasonable to hypothesize that the capacity of high-dose folate to suppress the pro-inflammatory effects of LPS reflects its ability to antagonize the up-regulatory impact of ROS on LPS signaling pathways.

Moreover, in light of high-dose folate's ability to scavenge peroxynitrite-induced radicals, it is pertinent to note that peroxynitrite plays a key role in the organ damage induced by sepsis; a number of studies in rodent models of sepsis demonstrate that administration of peroxynitrite decomposition catalysts can markedly decrease mortality and lessen damage to vital organs – liver, gut, lung, kidney, and circulatory system.[145-148] Hence, since reduced folates can scavenge peroxynitrite-derived radicals, and since folate's efficacy in this regard is thought to account for its ability to restore coupling of eNOS, it has been suggested that high-dose folate might be clinically useful in sepsis by lessening the toxicity of peroxynitrite.[137]

ERK1/2 Activation Promotes Tissue Factor Expression – Impact of AMPK Activators

The mechanism whereby LPS-mediated TLR4 activation stimulates ERK1/2 activity is still rather obscure; some studies suggest that Ras isoprenylation and activation may mediate this pathway.[149] While ERK1/2 can promote AP-1 signaling, it also contributes importantly to increased expression of tissue factor (TF) and TNF-alpha by boosting transcription of early growth response factor-1 (Egr-1).[150] Egr-1 binds to the promoters of the TF and TNF-alpha genes, promoting their transcription. Increased TF expression is a mediator of the disseminated intravascular coagulation (DIC) associated with sepsis.[151]

It is not clear whether glycine or inhibitors of NADPH oxidase could influence TLR4-mediated activation of ERK1/2. A report that inhibition of CaMKII suppresses LPS-mediated activation of ERK1/2 in macrophages[123] can be interpreted as evidence that glycine might be useful in this regard. Furthermore, folate dose-dependently inhibited ERK1/2 phosphorylation in LPS-treated macrophages, suggesting that ROS may indeed promote ERK1/2 activation – and that high-dose folate may have potential for suppressing DIC in sepsis.[132]

Curiously, metformin may also have potential in this regard. Although a clinical concentration of metformin (10 μM) did not influence LPS-mediated activation of NF-kappaB, p38 MAP kinase, or JNK in human monocytes, it suppressed ERK1/2 activation and up-regulation of TF and TNF-alpha expression.[152] This study did not determine whether AMPK mediated this effect; however, another clinical activator of AMPK, berberine, has been reported to suppress expression of TF by LPS-stimulated macrophages, in clinically pertinent sub-micromolar levels.[153] Metformin was reported to reduce mortality in mice injected with LPS, but not with E.coli.[154, 155] Metformin treatment protected the liver when endotoxin was administered to partially-hepatectomized rats.[156] Analogously, berberine protected rodents from LPS in several studies – albeit this poorly absorbed agent was administered parenterally in these studies.[157-161]

Mechanisms by which PhyCB, glycine, high-dose folate, and metformin/berberine may oppose TLR4-mediated activation and pathogenicity of macrophages/monocytes are depicted in Figure 1.

Preventing Vascular Hyperpermeability

Vascular hyperpermeability is a key feature of the pathogenesis of septic shock; protein extravasation leads to edema, and also promotes vascular hypovolemia, exacerbating the impact of iNOS on hypotension.[162] LPS directly, or pro-inflammatory cytokines released from macrophages such as TNF-alpha, can act on endothelial cells to impair the expression and appropriate function of proteins such as VE-cadherin, occludin, and claudin-5 required for formation of interendothelial adherens and tight junctions. Pertinently, these effects have been shown to require activation of NADPH oxidase complexes, as agents which inhibit NADPH oxidase or block its expression have been shown to suppress LPS/cytokine-mediated endothelial hyperpermeability.[163-167] The acute effect of increased superoxide production on endothelial junctions has been shown to be mediated by peroxynitrite.[165] This chronically up-regulates the activity of protein phosphatase 2A (PP2A) by inducing a tyrosine nitration that prevents its inhibition by tyrosine kinases; PP2A dephosphorylates tight junction proteins, suppressing their function. In the longer term, ROS generated by NADPH oxidase down-regulate the expression of key proteins required for tight junction and adherens junction formation.[167]

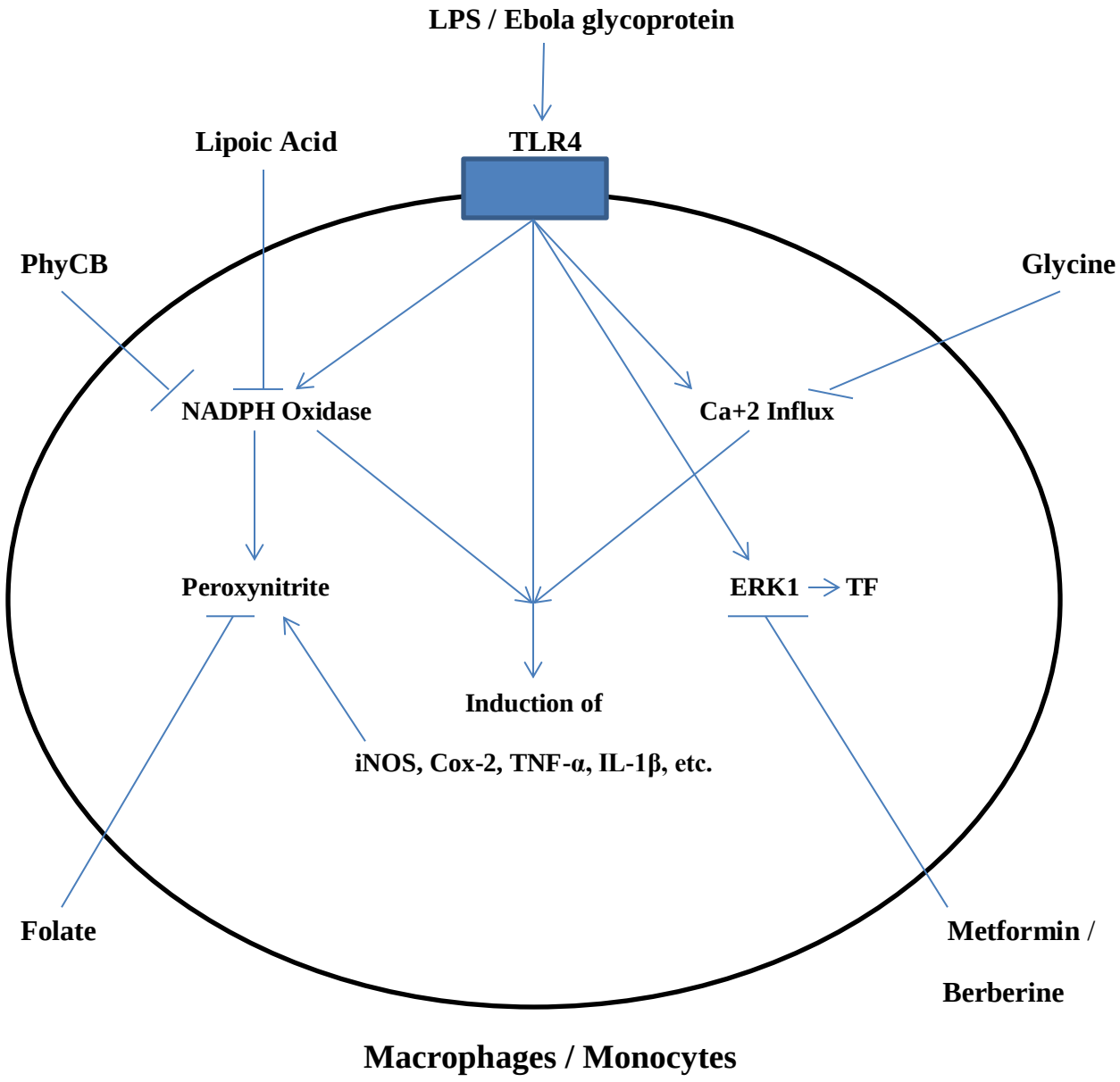

Figure 1. Strategies for Opposing TLR4-Mediated Activation of Macrophages / Monocytes

Conversely, AMPK activity promotes tight junction formation in vascular endothelium and in endothelia, and opposes the hyperpermeability induced by LPS and TNF-alpha.[168-175] In part, this reflects AMPK-

mediated suppression of angiopoietin-2 expression; the latter boosts the responsiveness of endothelial cells to TNF-alpha and other inflammatory cytokines.[174, 176, 177]

It is therefore reasonable to predict that biliverdin or PhyCB could help to maintain vascular integrity during septic infection by moderating NADPH oxidase activation. Moreover, since peroxynitrite appears to be a key mediator of this adverse impact of sepsis on interendothelial junctions, the ability of high-dose folate to quench peroxynitrite-derived radicals suggests that it also could be helpful in this regard. It is less clear whether glycine would impact endothelial permeability. Not surprisingly, the AMPK activators metformin and berberine have shown favorable effects on tight junction formation in endothelia and epithelia;[171, 173, 178-181] hence, they may have potential for preventing vascular hyperpermeability during sepsis.

Pertinence to Ebola Infection

The pathogenesis of Ebola infection, whose chief cellular targets are macrophages, monocytes, and dendritic cells, is strikingly parallel to that of septic shock; it is characterized by a massive increase in pro-inflammatory cytokines, hypotension, vascular hyperpermeabillity, and DIC.[182] This likely reflects the fact that the membrane glycoprotein in Ebola virions, and in the virus-like particles shed by infected macrophages/dendritic cells, is a potent agonist for the TLR4-MD2 receptor, just like LPS.[183, 184] Curiously, although Ebola infection boosts cytokine production in a manner analogous to LPS, the viral proteins V24 and V35 act in diverse ways to suppress the production and activity of type I interferons, while also inhibiting the capacity of dendritic cells to act as effective antigen-presenting cells.[185, 186] Moreover, Ebola infection causes apoptotic death of bystander lymphocytes, possibly by up-regulating receptor of the death receptor ligand TRAIL.[187] Hence, by suppressing both innate and acquired mechanisms of anti-viral resistance, Ebola typically gives rise to persistent infection which induces a sustained and worsening sepsis-like state. The intense vascular hyperpermeability induced by Ebola appears to reflect the joint impact on endothelium of TNF-alpha and of virus-like microparticles bearing the Ebola membrane glycoprotein.[188] As in sepsis, increased expression of TF by macrophages and monocytes appears to trigger DIC.[189]

Until drugs or anti-sera that can directly target Ebola are available, the best approach to treating Ebola infection is to control the sepsis-like syndrome it gives rise to. There is good reason to suspect that the measures recommended above for control of sepsis would likewise be beneficial in Ebola infection. Moreover, there is a recent report that induction of heme oxygenase-1 (HO-1) slows the replication of Ebola within host cells; the authors did not determine which of the products of HO-1 activity, carbon monoxide or biliverdin, mediates this protective effect.[190] If the latter is responsible, administration of biliverdin and perhaps of PhyCB might have potential for slowing the spread of Ebola within the body, perhaps giving the immune system a better chance to cope with it. Evidently, the HO-1 inducer LA may have clinical potential in this regard.

Overview

LPS-triggered activation of NADPH oxidase complexes has an up-regulatory impact on the signaling pathways which mediate endotoxin's pro-inflammatory effects; biliverdin and its phycochemical homolog PhyCB, which can inhibit such complexes, hence may have therapeutic potential in sepsis. Glycine would be expected to complement the NADPH oxidase-inhibitory activity of moderate doses of biliverdin/PhyCB, while at the same time opposing other pro-inflammatory signaling pathways mediated by the LPS-triggered increase in Ca_i. Folate's antioxidant activity may antagonize the up-regulatory effects of oxidants on pro-inflammatory cell signaling, and in particular lessen the pathogenic impact of peroxynitrite. AMPK activators such as metformin or berberine may lessen risk for DIC; these agents, as well as biliverdin/PhyCB and high-dose folate, could act directly on endothelial cells to prevent vascular hyperpermeability. While it may prove technically feasible to develop new drugs that aid survival in endotoxemia by targeting specific signaling intermediates, biliverdin/PhCB, glycine, folate, and metformin/berberine have the considerable advantage that they are natural compounds or venerable drugs known to be safe and reasonably well tolerated, and that indeed may have versatile health-protective properties.[73, 129, 137, 192-196]

It should however be acknowledged that, to the extent that these agents can blunt inflammation in the context of gram-negative infection, they may to some degree lessen the efficacy of the immune response for clearing the infectious agent. Hence, any such measures would be expected to achieve their greatest net benefit in the context of aggressive and well-chosen antibiotic therapy.

Dedication

This paper is dedicated to the memory of my father, Frederick Briggs McCarty.

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