Potential Utility of Parenteral Phycocyanobilin in Ischemia-Reperfusion Injury and Other Acute Syndromes Associated with Severe Oxidative Stress

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Abstract

Oxidative/nitroxidative stress is a key mediator of the cellular death and dysfunction associated with ischemia-reperfusion injury and with various acute medical emergencies characterized by circulatory shock and multiple organ failure. Activated NADPH oxidase and oxidatively-damaged mitochondrial respiratory chains are primary sources of excessive superoxide generation in these syndromes, with subsidiary contributions made by xanthine oxidase and uncoupled eNOS. Phycocyanobilin (PhyCB), the chief phytochemical in spirulina, can function as a potent inhibitor of NADPH oxidase, mimicking the physiological activity of biliverdin/bilirubin in this regard. Thus, parenteral PhyCB may have considerable potential as an adjuvant to thrombolytic therapy of heart attack and stroke, and in the management of other oxidative stress emergencies such as acute respiratory distress syndrome, hemorrhagic or septic shock, and severe burn injury. However, since PhyCB will not directly inhibit superoxide production by damaged mitochondria or xanthine oxidase, it would be rational to administer PhyCB in conjunction with other agents which have the potential to quell the downstream adverse consequences of superoxide excess. In this regard, high-dose folates have the potential to protect cells from peroxynitrite-derived radicals, and ethyl pyruvate promotes scavenging of hydrogen peroxide. Thus, a parenteral “rescue solution” providing PhyCB, high-dose folate, and ethyl pyruvate would be expected to be a worthwhile adjunct to thrombolytic therapy, and to have utility in the management of other acute oxidative stress emergencies.

Role of Oxidative/Nitroxidative Stress in Ischemia-Reperfusion Injury

There is a wealth of evidence that excessive oxidative stress is a key mediator of the tissue damage evoked by ischemia-reperfusion (IR) injury. In tissues which express constitutive NO synthase activity, or in which inflammation has induced iNOS activity, nitroxidative stress (i.e. peroxynitrite-derived radicals) can also contribute importantly to cellular death and dysfunction.

Important sources of this oxidative stress can include oxidatively-damaged mitochondrial respiratory chains, activated NADPH oxidase, and xanthine oxidase. Remarkably, hypoxia (as opposed to anoxia) has been shown to boost superoxide production by mitochondria; the resulting disruption of the mitochondrial inner membrane impairs the efficiency of respiratory electron flow, such that mitochondria become proficient sources of superoxide once oxygenation is restored.\(^1,2\) Increased activation of NADPH oxidase is often seen following re-oxygenation, for reasons that remain rather obscure, but that appear to be contingent on Rac-1 activation and increased expression of NAPDH oxidase subunits.\(^3,5\) Oxidative stress or increased proteolytic enzyme activity may also contribute to conversion of xanthine dehydrogenase to xanthine oxidase, which can further boost superoxide production in re-oxygenated tissue;\(^6,7\) in coronary endothelial cells, post-ischemic xanthine oxidase activation is a downstream consequence of enhanced NADPH oxidase activity.\(^8\) Oxidatively-uncoupled eNOS may also become a
source of superoxide in endothelial cells.\textsuperscript{9-11} Perversely, there are a number of feedforward mechanisms whereby increased oxidative stress derived from one source can evoke increased production of oxidants by other sources.

Whereas pre-treatment with certain lipophilic antioxidants or with agents that boost intracellular glutathione levels can limit the adverse impact of IR on mitochondrial oxidant generation by helping to preserve the proper structure and function of the respiratory chain,\textsuperscript{2, 12, 13} there is no clear path to correcting this damage in the post-ischemic period. In contrast, both NADPH oxidase and xanthine oxidase are susceptible to rapid inhibition by certain drugs, which thus have potential for the clinical management of IR injury.

Increased activity of NADPH oxidase appears to be a key mediator of IR injury in many tissues, for several reasons. Activation of NADPH oxidase in endothelial cells in the post-ischemic region, or in penumbral regions, exerts pro-inflammatory effects that can increase tissue permeability and promote the influx of neutrophils and other leukocytes, while also impairing microcirculatory flow.\textsuperscript{4, 5, 14-17} Moreover, tissue damage tends to elevate NADPH oxidase activity in neutrophils, and the oxidative stress contributed by influxing neutrophils is often an important mediator of tissue damage in IR injury; indeed, neutrophils appear to be the primary mediators of damage to tissues distant from the site of ischemia.\textsuperscript{18-20} Furthermore, IR often evokes increased NADPH oxidase activity in parenchymal cells – including cardiomyocytes and cerebral neurons – which likewise may contribute to the death and dysfunction of these cells.\textsuperscript{21-24} It is therefore not surprising that agents capable of inhibiting NAPDH oxidase activity – including apocynin and DPI – have often been found to confer considerable protection from IR injury to the brain, heart, lung, liver, and kidney in rodent studies.\textsuperscript{4, 5, 8, 16, 21, 22, 25-31}

**Phycocyanobilin – a Natural Inhibitor of NADPH Oxidase**

The recent discovery that free bilirubin can function physiologically as an inhibitor of NADPH oxidase activity,\textsuperscript{32-35} helps to rationalize several studies showing that infusion of bilirubin or its immediate precursor biliverdin can often provide protection from IR injury;\textsuperscript{36-45} moreover, these agents are likely to be at least partly responsible for the protection afforded by induction of heme oxygenase-1 – one of whose chief products is biliverdin. However, in a few studies examining IR in the kidneys, bilirubin was only notably protective if accompanied by increased delivery of carbon monoxide (another key product of HO-1).\textsuperscript{46, 47}

It is now known that phycocyanobilin (PhyCB), a biliverdin derivative that is the chief phytounutrient found in spirulina, can mimic the inhibitory impact of biliverdin/bilirubin on NADPH oxidase activity.\textsuperscript{48} This likely accounts for the remarkably versatile anti-inflammatory and cytoprotective effects of oral or parenteral phycocyanin (the spirulina protein which contains PhyCB as a chromophore) demonstrated in rodents.\textsuperscript{48, 49} Thus, it is pertinent to note that perfusion with phycocyanin in a Langendorff heart model of IR injury was shown to alleviate the extent of infarction and aid cardiac functional recovery.\textsuperscript{50} Presumably, lysosomal degradation of phycocyanin liberates free PhyCB or PhyCB-cysteiny1 oligopeptides capable of inhibiting NADPH oxidase after reduction by biliverdin reductase.

Inasmuch as PhyCB, which constitutes about 0.6% of the dry weight of spirulina, is readily available, and moreover is the subject of a pending patent covering its use as a nutraceutical or parenterally-
administered drug, it may be feasible to develop PhyCB as a drug that could be administered intravenously or intra-arterially as an adjunct to thrombolytic therapy of MI or stroke, with the intent of minimizing IR-induced injury to the heart or brain. Parenteral PhyCB would also likely be useful in other emergency medical settings in which excessive oxidative/nitroxidative stress is a prominent mediator of tissue damage and mortality – possibly including septic shock, severe hemorrhage, burn injury, acute respiratory distress syndrome and multi-organ failure syndrome. In this regard, administration of either bilirubin or biliverdin has been found to be protective in rodent models of septic shock – in part by blunting induction of iNOS. Not surprisingly, apocynin can also provide protection in rodent models of sepsis.

A “Rescue Solution” for Acute Oxidant Stress Syndromes

For best clinical results, PhyCB could be administered in conjunction with other safe agents which would be expected to have a complementary impact on oxidative/nitroxidative stress. The use of such agents appears advisable in light of the fact that, whereas PhyCB would be expected to quell superoxide production stemming from NADPH oxidase, there is no reason to suspect that it would have any direct impact on superoxide production by damaged mitochondria, xanthine oxidase, or uncoupled eNOS (albeit it might act indirectly, by intervening in the feed-forward mechanisms that activate these sources of superoxide). The pathogenic effects of excessive superoxide are mediated primarily by its metabolic products peroxynitrite and hydrogen peroxide. There is recent evidence that reduced forms of the B vitamin folate can efficiently scavenge peroxynitrite-derived radicals; moreover, the natural metabolite pyruvate – most effectively administered as its more stable ester, ethyl pyruvate – can directly scavenge hydrogen peroxide. Thus, high-dose folate and ethyl pyruvate may have particular potential as adjuncts to PhyCB in the management of the acute syndromes driven by excessive oxidative/nitroxidative stress. High-dose folate or folinic acid (leucovorin) can be accumulated to high concentrations in endothelial cells and other cell types. Intracellular concentrations of folates within endothelial cells can increase by two orders of magnitude when exposed to elevated extracellular concentrations of this vitamin. (In contrast, intracellular levels of the versatile antioxidant ascorbate are virtually saturated at high-physiological serum concentrations, and clinically tolerable doses of N-acetylcysteine have only a modest impact on normal intracellular glutathione levels. Within cells, folates are rapidly reduced to tetrahydro forms, and these can function efficiently as scavengers of radicals – most notably, peroxynitrite-derived radicals. This likely explains the remarkably favorable impact of high-dose folate in a rodent model of cardiac IR, as well as the re-coupling effect of high-dose folate (or reduced folates) on eNOS activity in inflamed endothelium. Peroxynitrite-derived radicals, in part via activation of PARP, appear to play a key role in the cellular death and dysfunction seen in critical medical disorders characterized by severe oxidative stress and inflammation, such as septic or hemorrhagic shock and burn injury. (These findings also indirectly suggest the utility of NADPH oxidase inhibition in these disorders, since superoxide derived from activated NADPH oxidase likely is a key precursor of the peroxynitrite evolved in these circumstances.) High-dose folate could be administered as folic acid, as 5-methyltetrahydrofolate (the chief circulating form of folate in normal physiology), or as folinic acid – a.k.a. leucovorin, already approved for intravenous administration as an adjuvant to certain cancer chemotherapies.
Another agent with great promise in this context is ethyl pyruvate. Pyruvate and certain other alpha-keto acids can interact directly with hydrogen peroxide to quench it; the reaction between pyruvate and hydrogen peroxide yields harmless acetate, carbon dioxide, and water.\(^{74}\) During oxidative stress, hydrogen peroxide, by oxidizing sulfhydryl groups in signaling molecules, promotes pro-inflammatory signaling; additionally, in the presence of free transition metals, it can be reduced to yield the hyperreactive hydroxyl radical. Thus, pyruvate has considerable antioxidant potential – as has been confirmed in a number of animal studies\(^{75-79}\) – but its clinical feasibility in this regard is somewhat compromised by the fact that high concentrations of pyruvate are unstable in aqueous solution, owing to condensation reactions which yield parapyruvate, a mitochondrial toxin.\(^{80}\) Hence, if used clinically, it would be desirable to solubilize it shortly prior to infusion.

For this reason, it has been proposed that pyruvate should be administered as stable esters such as ethyl pyruvate, which can evolve pyruvate gradually owing to plasma or intracellular esterase activity.\(^{81,82}\) Moreover, ethyl pyruvate has proven to have additional advantages – it is relatively lipophilic, giving it improved access to intracellular compartments; also, it has a durable anti-inflammatory impact that reflects its ability to alkylate a cysteine group in the p65 subunit of NF-kappaB, blocking its ability to bind to DNA and thereby regulate transcription. The utility of ethyl pyruvate in rodent models of IR or septic shock has been documented.\(^{83-90}\) Parenteral ethyl pyruvate has proven to be well tolerated in initial clinical studies. A phase II clinical study with ethyl pyruvate as an adjuvant for cardiopulmonary bypass surgery failed to demonstrate clinical benefit.\(^{91}\) However, this study would be unlikely to reveal ethyl pyruvate’s antioxidant potential, since this agent, which has a short plasma half-life owing to metabolic consumption, was not administered continuously, but rather 1 hour every 6 hours during the day of surgery; this protocol presumably would have achieved repression of NF-kappaB activity, but inflammatory response was relatively muted in the control group, so perhaps little clinical benefit should have been expected under these circumstances. In any case, it is clear that a continuous infusion of ethyl pyruvate (or of sodium pyruvate), in an amount sufficient to sustain millimolar arterial plasma concentrations, would be required to optimize its hydrogen peroxide-quenching potential.

It can thus be predicted that a “rescue solution” providing PhyCB, high-dose folate, and ethyl pyruvate – all of which can be expected to be quite safe within clinically useful dose levels - could have a remarkably favorable impact on the contribution of oxidative stress to the tissue damage and mortality associated with post-ischemic injury to the heart, brain, and other tissues, and with various life-threatening syndromes leading to multi-organ failure such as septic shock, severe burn injury, hemorrhagic shock, and acute respiratory distress syndrome. Whether adjunctive use of additional antioxidant agents – such as allopurinol, N-acetylcysteine, or deoxyascorbate\(^{92,95}\) – could meaningfully boost the efficacy of such a regimen, could be evaluated in experimental models. Deoxyascorbate is of particular interest, as, owing to efficient intracellular uptake mediated by equilibrative glucose transporters, it has the potential to increase cellular ascorbate levels several-fold, as rapid intracellular reduction of dehydroascorbate effectively traps the evolved ascorbate within the cell. (In contrast, cellular uptake of ascorbate per se is already maximized at high-physiological plasma levels.\(^{59}\))
A Postscript on NF-kappaB and Salicylate

One of the major mediators of the pathogenic impact of oxidative stress and pro-inflammatory cytokines during ischemia-reperfusion or shock syndromes is NF-kappaB. Activation of this transcription factor boosts neutrophil influx into affected tissues, while increasing production of various cytokines, enhancing expression of iNOS, and, in some circumstances, promoting apoptosis. On the other hand, constitutive physiological activity of NF-kappaB can play a protective role, by increasing the expression of various antioxidant and anti-apoptotic proteins; this rationalizes a key role for NF-kappaB activation in pre-conditioning strategies that offer protection from subsequent ischemia-reperfusion. These considerations suggest that temporary inhibition of NF-kappaB activation, in the hours immediately following an ischemic insult, might well protect post-ischemic tissue – and sometimes distant tissues such as lung and the heart – from inflammatory damage. Indeed, protection of heart, brain, liver, kidney, skeletal muscle, stomach, and intestinal mucosa from direct or indirect ischemia-reperfusion damage has been demonstrated in rodents treated with a variety of measures which suppress NF-kappaB activity in the hours following reperfusion.

The most readily available clinical agent for suppressing NF-kappaB activation is the drug sodium salicylate, which blocks activation of NF-kappaB via many (though not all) stimuli by impeding activation of the upstream kinase IKKbeta. Over a decade ago, Zund and colleagues demonstrated that high-clinical concentrations of salicylate could inhibit induction of ICAM-1 in hypoxic endothelial cells exposed to LPS; these authors suggested that salicylate could be used to prevent ischemia-reperfusion damage to the heart during cardiopulmonary bypass. Other experimental agents which target IKKbeta have likewise been shown to be protective in rodent models of ischemia-reperfusion. Most notably, inhibition of IKKbeta commencing 2 hours after the start of reperfusion reduced infarct area by over 50% in a mouse model of myocardial ischemia-reperfusion. A recent clinical study in overweight volunteers reveals that a standard clinical dose of sodium salicylate – i.e. 4.5 g daily in three divided doses as salsalate – can indeed diminish NF-kappaB activation in vascular endothelium. However, salicylate spares moderate constitutive NF-kappaB activity that protects cells from apoptosis and oxidant stress; this presumably explains why, in oxidant-stressed rat neuronal cultures, salicylate proved to be protective, whereas a drug which blocked nuclear translocation of NF-kappaB exacerbated cell damage. Since salicylate is not available as a parenteral drug, it would need to be administered orally as soon as possible after symptoms of an infarction; pharmacokinetic studies would be required to determine what tolerable loading dose could enable clinically useful plasma concentrations (around 20 mg/dl) to be achieved rapidly. In light of a report that a single dose of 1,074 mg sodium ascorbate produces a peak plasma concentration of 84 mg/dl after 45 minutes – falling to about 60 mg/dl after 3 hours – it seems likely that a loading dose of 3 g, followed 3 hours later by another 1.5 g, might achieve safe, clinically useful plasma levels in the critical hours following reperfusion. Such a strategy might prove to be a worthwhile complement to the antioxidant strategies suggested above.


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