

openheart Thrombotic complications of COVID-19 may reflect an upregulation of endothelial tissue factor expression that is contingent on activation of endosomal NADPH oxidase

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To cite: DiNicolantonio JJ, McCarty M. Thrombotic complications of COVID-19 may reflect an upregulation of endothelial tissue factor expression that is contingent on activation of endosomal NADPH oxidase. *Open Heart* 2020;**7**:e001337. doi:10.1136/openhrt-2020-001337

Accepted 27 May 2020

ABSTRACT

The high rate of thrombotic complications associated with COVID-19 seems likely to reflect viral infection of vascular endothelial cells, which express the ACE2 protein that enables SARS-CoV-2 to invade cells. Various proinflammatory stimuli can promote thrombosis by inducing luminal endothelial expression of tissue factor (TF), which interacts with circulating coagulation factor VII to trigger extrinsic coagulation. The signalling mechanism whereby these stimuli evoke TF expression entails activation of NADPH oxidase, upstream from activation of the NF-kappaB transcription factor that drives the induced transcription of the TF gene. When single-stranded RNA viruses are taken up into cellular endosomes, they stimulate endosomal formation and activation of NADPH oxidase complexes via RNA-responsive toll-like receptor 7. It is therefore proposed that SARS-CoV-2 infection of endothelial cells evokes the expression of TF which is contingent on endosomal NADPH oxidase activation. If this hypothesis is correct, hydroxychloroquine, spirulina (more specifically, its chromophore phycocyanobilin) and high-dose glycine may have practical potential for mitigating the elevated thrombotic risk associated with COVID-19.

A KEY ROLE FOR ENDOSOMAL NADPH OXIDASE IN ENDOTHELIAL TISSUE FACTOR EXPRESSION

COVID-19 is associated with a high incidence of thrombotic complications.¹ Necropsy studies reveal platelet-fibrin plugs in pulmonary arterioles, likely contributing to the hypoxaemia characteristic of advanced infection.² It has been suggested that the thrombotic diathesis associated with COVID-19 reflects an endotheliopathy induced by viral infection of endothelial cells.^{3–5} These cells prominently express the ACE2 plasma membrane protein to which the spike protein of SARS-CoV-2 virions bind, enabling their endosomal incorporation into cells.^{6,7} The thrombotic complications of COVID-19 infection would be readily explained if SARS-CoV-2 infection of endothelial cells induces luminal expression of tissue

factor (TF), which could then interact with circulating coagulation factor VII to trigger a proteolytic cascade culminating in the generation of thrombin and fibrin (extrinsic clotting). TF expression is negligible in healthy non-inflamed endothelial cells, but it can be upregulated at the transcription level by various proinflammatory stimuli that activate the NF-kappaB transcription factor. More specifically, the heterodimers p65/p50 or p65/c-Rel can bind to a novel ‘TF-kappaB’ sequence in the promoter of the TF gene, driving its induced expression.^{8–10}

Various proinflammatory factors that induce TF in endothelial cells—including tumour necrosis factor-alpha (TNF α), antiphospholipid antibodies (aPL), ultrafine pollutant particles and homocysteine—have been shown to do so via signalling pathways in which activation of NADPH oxidase complexes plays an obligate role.^{11–14} The effects of aPL and of TNF α in this regard hinge on the activation of NADPH oxidase in endosomes which has incorporated these agonists.¹²

Prior to the emergence of SARS-CoV-2, it was demonstrated that a number of RNA viruses can activate endosomal NADPH oxidase through a mechanism dependent on toll-like receptor 7 (TLR7), which is activated by binding to single-stranded RNA.¹⁵ Presumably, these viruses, after binding to cellular plasma membranes, are incorporated into endosomes, and viral RNA released from the virions can interact with endosomal TLR7, triggering NADPH oxidase activation. Indeed, To and colleagues found that eight different types of single-stranded RNA viruses activated endosomal NADPH oxidase in alveolar macrophages, and the two types that did not activate it do not employ endosomes as their primary entry mechanism.¹⁵ Moreover, this effect was



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absent in alveolar macrophages in which TLR7 expression was knocked out.

SARS-CoV-2 is likewise a single-stranded RNA virus, the intracellular uptake of which is mediated by endosomes.¹⁶ We postulated that SARS-CoV-2, after incorporation into endosomes within endothelial cells, can likewise activate endosomal NADPH oxidase via TLR7, and that the resulting local production of superoxide/hydrogen peroxide leads to activation of NF-kappaB—by a mechanism yet to be determined—and subsequently to increased expression of TF. At present, while it is difficult to trace clinical studies that have measured serum markers of oxidative stress in COVID-19 patients, the fact that clinical outcomes were poorer in those provinces of China where soil selenium is deficient is compatible with the view that oxidant stress plays a key pathogenic role in this syndrome, and selenium is required for function of multiple antioxidant enzymes, including glutathione peroxidases and thioredoxin reductases.^{17 18} Moreover, oxidative stress is a key feature of other viral diseases that evoke acute respiratory distress syndrome and cytokine storm.¹⁹

THERAPEUTIC IMPLICATIONS OF THE HYPOTHESIS

This hypothesis is of practical significance because practical measures for inhibiting endosomal NADPH activity may be at hand. Hydroxychloroquine (HCQ), the antimalarial agent now commonly used to treat systemic lupus erythematosus (SLE) and rheumatoid arthritis, has been shown to decrease the elevated risk for thrombotic complications associated with SLE.^{20 21} It has recently been demonstrated that HCQ, likely via its alkalinising effect on endosomes, abrogates the ability of aPL to activate endosomal NOX2-dependent NADPH oxidase activity, and this reflects a failure of NOX2 to be translocated into endosomes.²² The authors further demonstrated that HCQ treatment protects mice from aPL-induced thrombosis. We postulated that HCQ can likewise prevent endosomal NADPH oxidase activation in SARS-CoV-2-exposed endothelial cells, thereby reducing risk for the thrombotic complications associated with COVID-19 infection. This is of particular interest in light of the ability of HCQ to inhibit SARS-CoV-2 *in vitro*, and of preliminary evidence that administration of HCQ early in the course of COVID-19 may improve therapeutic outcomes, likely by slowing cell-to-cell spread of the virus.^{23–25} HCQ-mediated alkalination of endosomes could be expected to suppress the proteolytic activity of cathepsin L, required for endosomally entrapped SARS-CoV-2 virions to escape into the cytoplasm.^{16 26 27}

In addition, phycocyanobilin (PCB), a biliverdin metabolite prominently expressed as a light-absorbing chromophore in cyanobacteria (such as spirulina) and many blue-green algae, has been found to mimic the ability of its chemical relative unconjugated bilirubin to inhibit NADPH oxidase complexes.^{28 29} This likely explains the strong antioxidant/anti-inflammatory effects of orally administered spirulina (or of phycocyanin, the spirulina protein to which PCB is covalently attached) in a number of rodent models

of heart disorders in which NADPH oxidase activation plays a pathogenic role.^{28 30} It is therefore proposed that oral administration of PCB (or of spirulina or of phycocyanin) could help prevent thrombotic complications of COVID-19. Independent considerations suggested that this agent might also be useful for boosting the type 1 interferon response to SARS-CoV-2 and other RNA viruses and for blunting the cytokine storm that complicates late-stage COVID-19.³¹

Theoretical considerations suggested that elevated plasma levels of the amino acid glycine—which is known to have intriguing anti-inflammatory properties—may suppress endosomal activation of NADPH oxidase in cells that express strychnine-inhibitable glycine receptors.³² Indeed, endothelial cells express such receptors.^{33 34} Hence, the impact of supplemental glycine—inexpensive, well-tolerated and pleasantly sweet—on TF expression in endothelial cells exposed to proinflammatory stimuli—including SARS-CoV-2—would be of interest to study. Glycine supplementation might also aid control of thrombotic complications via a direct antiaggregatory effect on platelets mediated by glycine receptors.³⁵

The upregulatory impact of NADPH oxidase activity on NF-kappaB activation and TF expression is likely mediated by reversible oxidation by hydrogen peroxide of sulfhydryl groups in specific proteins that participate in this activation.^{36 37} If so, then boosting synthesis of glutathione and thioredoxin while upregulating the expression of enzymes that work with them to reverse cysteine oxidations (eg, glutathione reductase and thioredoxin reductase) would be expected to counteract the impact of hydrogen peroxide on NF-kappaB activation.³⁸ Moreover, increasing the expression of glutathione peroxidase would also counteract the signalling impacts of hydrogen peroxide. These benefits could be achieved with clinically useful phase 2 inducer nutraceuticals—such as lipoic acid, ferulic acid or sulforaphane—complemented by supplementation with N-acetylcysteine; the latter supplies cysteine, the rate-limiting substrate for glutathione synthesis.^{39–46} Glycine supplementation can also promote glutathione synthesis.^{47 48}

A number of studies have observed that nitric oxide (NO) and endothelial NO synthase (eNOS) activity oppose the endothelial induction of TF by various proinflammatory stimuli. The ability of drugs that stimulate or activate soluble guanylate cyclase (sGC) to replicate this effect suggests that cGMP is the downstream mediator of NO's impact in this regard.⁴⁹ Intriguingly, these drugs do not influence the ability of TNF α to suppress protein levels of IkappaB, but nonetheless they do suppress the transcriptional activity of NF-kappaB as assessed with a transfected reporter gene.⁴⁹ Since the oxidative stress induced by NADPH oxidase activation would seem likely to promote uncoupling of eNOS, agents that correct this uncoupling might be expected to downregulate COVID-19-mediated TF induction. Specifically, supplemental intakes of citrulline and of high-dose folate might be useful in this regard, as these agents counteract the uncoupling induced by asymmetric dimethylarginine and by oxidation of

tetrahydrobiopterin, respectively.^{50–53} Alternatively, agents that directly stimulate or activate sGC might also be useful for suppressing TF induction; in addition to pharmaceuticals that have this effect, supraphysiological concentrations of biotin can stimulate sGC activity.^{54 55}

Measures that quell endothelial oxidative stress while supporting effective eNOS activity might not only help to control the thrombotic complications of COVID-19, but also be expected to blunt the exuberant influx of neutrophils that promote respiratory distress in this syndrome. Nitric oxide, by both cGMP-dependent and cGMP-independent mechanism, inhibits endothelial expression of adhesion factors for neutrophils whereas oxidants upregulate such expression.^{56–67}

Box 1 proposes dose schedules for the drugs/nutraceuticals cited earlier that might have practical potential for reducing the thrombotic risk associated with COVID-19. These suggestions deal with agents that might impact the endothelial activation associated with COVID-19. Evidently, drugs that address overactive coagulation or platelet activation may also have potential for controlling the thrombotic complications of this syndrome; in that regard, many physicians are currently employing heparin injections.^{68 69}

LIMITATIONS

This essay proposes a hypothesis that, in the authors' opinion, is credible and, if true, it should help to explain the common thromboembolic complications of COVID-19, while also suggesting practical measures that could lessen the thrombogenicity of vascular endothelium infected by the virus, or exposed to proinflammatory cytokines released in response to viral infection. It is intended to stimulate preclinical study of interactions between SARS-CoV-2 and vascular endothelial cells which could be useful for affirming or disproving the hypothesis. This hypothesis should not be considered to be proven, and the suggestions it provides regarding drugs or nutraceuticals which might ameliorate vascular dysfunction during COVID-19 should not be considered as prescriptive.

Contributors All authors contributed to the final manuscript.

Box 1 Suggested dose schedules for drugs/nutraceuticals with antithrombotic potential in COVID-19

Hydroxychloroquine—200 mg, 2 times per day
 Spirulina—15 g (rounded tablespoon), one time per day
 Glycine powder—5 g, 2–3 times per day
 Lipoic acid—600 mg, 2–3 times per day
 Ferulic acid—500 mg, 2 times per day
 Broccoli sprout powder—5 g, 1–2 times per day (providing 20–40 mg of sulforaphane)
 N-acetylcysteine—600 mg, 2–3 times per day
 Citrulline powder—2 g, 2 times per day
 Folic acid—40 mg, one time per day
 Biotin—10 mg, 2–3 times per day

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests JJD is Director of Scientific Affairs at AIDP. MM is an owner of a nutraceutical company and co-inventor and co-owner of a US patent covering nutraceutical uses of phycocyanobilin oligopeptides derived from cyanobacteria such as spirulina.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; internally peer reviewed.

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