



Coping with endothelial superoxide: potential complementarity of arginine and high-dose folate

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Summary Superoxide overproduction is a prominent mediator of the endothelial dysfunction associated with a range of vascular disorders, acting in a number of complementary ways to inhibit effective endothelial nitric oxide (NO) activity. The ability of superoxide to quench NO is well known, but oxidants derived from superoxide also appear to inhibit dimethylarginine dimethylaminohydrolase (DDAH) and to oxidize tetrahydrobiopterin (THBP). The former effect boosts the level of methylated arginines that act as potent competitive inhibitors of NO synthase, whereas the latter effect decreases the ability of this enzyme to generate NO, while converting it to a form that readily generates superoxide. The adverse impact of DDAH deficiency on NO production can be offset with supplemental arginine. Although supplementation with THBP has the potential to compensate for the rapid oxidative destruction of this compound, and maintaining optimal vitamin C nutrition may protect or restore the endothelial THBP pool to a limited extent, the most practical way to optimize NO synthase activity in the context of THBP deficit may be administration of high-dose folic acid. The primary circulating metabolite of folate, 5-methyltetrahydrofolate (5MTHF), is structurally analogous to THBP, and appears to normalize the activity of NO synthase in THBP-depleted endothelial cells, either because it “pinch hits” for the absent THBP, or interacts allosterically with NO synthase in some other way to promote the proper function of this enzyme. This observation may rationalize recent clinical studies showing a favorable effect of oral folic acid (5–10 mg daily) on dysfunctional endothelium, independent of any concurrent modulation of homocysteine levels. A recent study reports that, whereas either arginine or THBP alone have only a modest impact on dysfunctional aortic endothelium derived from hypercholesterolemic mice, the combination of the two produces a complete normalization of endothelial function. In aggregate, these considerations suggest that joint administration of arginine and high-dose folate may represent a fruitful approach to preventing and treating vascular disorders – albeit the underlying overproduction of superoxide should also be addressed by ameliorating relevant vascular risk factors.
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Superoxide excess is a primary mediator of endotheliopathy

Excessive endothelial production of superoxide appears to be a key pathogenic factor in many vascular disorders characterized by endothelial dysfunction [1,2]. The chief source of this super-

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oxide appear to be membrane-bound NAD(P)H reductase [3–6], as well as “uncoupled” nitric oxide synthase (NOS) deficient in its cofactor tetrahydrobiopterin [7–9]; other enzymes that may contribute in this regard (most notably during ischemia/reperfusion) include xanthine oxidase and cyclooxygenase [10,11]. The pathogenicity of endothelial superoxide stems largely from the fact that it and its oxidant derivatives (peroxynitrite, hydrogen peroxide, hydroxyl radical) act in various ways to impede the production and bioactivity of endothelial nitric oxide (NO), which exerts crucial anti-inflammatory, anti-thrombotic, and anti-proliferative effects on the vasculature [12].

A central mediating role for superoxide excess in the endothelial dysfunction associated with many clinical disorders can be deduced from the fact that intravenous infusion of ascorbate – an effective scavenger of superoxide in the millimolar concentrations that can be achieved during intravenous administration [13] – improves NO-mediated endothelium-dependent vasodilation and/or basal NO production in a number of these conditions, including diabetes (both types), essential hypertension, hypercholesterolemia, hyperhomocysteinemia, angina, visceral obesity, smoking, aging, and acute free fatty acid excess [14–31].

DDAH and tetrahydrobiopterin as key targets of endothelial oxidant stress

The fact that superoxide reacts avidly with NO, thereby quenching the latter’s bioactivity, is well known [1]. However, endothelial oxidants work in other ways to compromise NO synthesis, while boosting their own production. Oxidant damage to the endothelial enzyme dimethylarginine dimethylaminohydrolase (DDAH) [32–34], the enzyme which catabolizes methylated arginines – asymmetric dimethylarginine (ADMA) and monomethylarginine (MAA) – that are potent competitive inhibitors of NOS [35,36], is now suspected to be largely responsible for the elevated plasma levels of ADMA typically seen in a wide range of endotheliopathies [37–40]. The resulting increase in intracellular ADMA can be expected to diminish NOS activity. The utility of dietary or parenteral arginine for improving endothelial function in many vascular disorders is thought to stem, at least in part, from the fact that an increase in intracellular arginine can offset the inhibition of NOS mediated by increased ADMA levels [41,42]. However, there is also some evidence that supplemental arginine can act to quell superoxide production – arginine

appears to have direct superoxide quenching activity [43], and it is conceivable that an increase in NO production induced by supplemental arginine – or possibly arginine itself – acts to down-regulate endothelial superoxide production [44], perhaps by impeding activation of NAD(P)H oxidase.

Another key target of superoxide and its downstream oxidant derivatives is tetrahydrobiopterin (THBP), an essential cofactor for NOS [8]. THBP is highly oxidant labile [45,46], and the favorable impact of THBP infusions on endothelial function in many types of endotheliopathies [47–56] is strong indirect evidence that THBP is diminished in endothelium that overproduces superoxide; peroxynitrite is especially active as an oxidant for THBP [57]. The turnover of endothelial THBP is quite rapid – levels in canine arteries *ex vivo* fall by 95% within 6 h after the rate-limiting enzyme for THBP synthesis, GTP cyclohydrolase (GCH), is inhibited [58]; presumably, the oxidant lability of THBP contributes importantly to this phenomenon [8]. Insulin resistance syndrome, commonly encountered in patients with vascular disorders, may exacerbate endothelial THBP deficiency by diminishing the expression of GCH [59,60].

In as much as THBP is crucial to efficient NOS function – for reasons not yet entirely clear – a deficiency of endothelial THBP decreases NO production. But there is a further noxious consequence – when NOS is sub-saturated with THBP, there is a steric change in the enzyme which renders it “uncoupled”, in the sense that high energy electrons from NADPH, which ordinarily interact with arginine and oxygen molecules to generate citrulline and NO, instead are routed directly to oxygen, generating superoxide [7–9]. In other words, as THBP deficiency worsens, NOS is converted progressively from a NO generator to a superoxide generator. This suggests the possibility of a vicious cycle, in which excess endothelial superoxide – perhaps stemming from NAD(P)H oxidase – diminishes the availability of THBP, thereby further boosting the production of superoxide (via NOS), which in turn further compromises THBP status. There is also reason to suspect that THBP deficiency may increase the requirement for arginine, inasmuch as the binding of THBP to the neuronal isoform of NOS increases the affinity of this enzyme for arginine [61,62]; thus, if it is correct to assume that this finding holds for the endothelial isoform as well, then a portion of the benefit of supplemental arginine treatment for endotheliopathies may reflect compensation for the diminished arginine affinity of NOS attributable to THBP deficiency.

Compensating for tetrahydrobiopterin loss – a surprising role for folic acid

Intracellular ascorbic acid acts to stabilize the endothelial THBP pool – either by protecting THBP from oxidant attack, or by reducing the oxidized derivatives of THBP back to tetrahydro form [45,46]. This may explain, at least in part, the favorable impact of high-dose *oral* ascorbate in various studies evaluating endothelium-dependent vasodilation [63–71] (inasmuch as oral ascorbate cannot produce the millimolar plasma levels required for it to scavenge superoxide in the presence of nitric oxide) [13,72]. Unfortunately, the protective scope of this vitamin is limited by the fact that endothelial uptake of ascorbate is maximized at a plasma concentration of about 100 μM [45,73], which is readily achieved by daily oral intakes of vitamin C of around 500 mg [63]. Thus, whereas maintaining good vitamin C status may be beneficial for preserving THBP bioactivity, oral mega-doses of vitamin C are unlikely to provide further benefit for endothelial health. Perhaps some of the vascular-protective benefits of phytochemical polyphenols stem from protection of THBP – this possibility requires evaluation.

An endothelial deficit of THBP can be rapidly corrected by parenteral administration of this cofactor – as reflected in the favorable impact of THBP infusion on endothelial function in various vascular disorders [47–52,54]. There is also a single study demonstrating that fairly ample oral intakes of THBP (10 mg/kg/day) can normalize the endothelial function of fructose-fed insulin-resistant rats [59]. Unfortunately, clinical use of oral THBP currently has limited feasibility, in light of the high cost of this chemical and the fact that its oxidant lability would mandate the use of high doses.

It is therefore extremely fortunate that folic acid – a far, far, cheaper compound – can somehow act to normalize NOS function in THBP-deficient endothelial cells [74]. The mediator of this beneficial effect appears to be the folate metabolite 5-methyltetrahydrofolate (5MTHF) [75,76], which is the major form of folic acid found in plasma – and indeed acute infusions of 5MTHF have been shown to have a favorable impact on dysfunctional endothelium [77–79]. When endothelial cells *in vitro* are depleted of THBP by treating them with inhibitors of GCH or exposure to homocysteine, superoxide production goes up, and NO production goes down; addition of THBP, 5MTHF, or even folic acid itself to the medium rapidly reverses this abnormality [76,80]. How

5MTHF acts to normalize the function of THBP-deficient NOS is not yet clear. Some researchers have presented evidence that 5MTHF may literally “pinch-hit” for THBP, binding to the same site on NOS as THBP and replicating its impact on the enzyme’s stereochemistry and activity [76]. However, other researchers report that 5MTHF fails to activate NOS is the complete absence of THBP, so there may be a more complex mechanism whereby 5MTHF supports effective NOS activity [75]. The possibility that 5MTHF may serve as a reductant for dihydrobiopterin, re-converting it to its active tetrahydro form, has also been suggested [81]. Whatever the explanation, there is definite evidence that 5MTHF tends to normalize the function of NOS when THBP is suboptimally available. The efficacy of folic acid in this regard may stem from its efficient conversion to 5MTHF.

The ability of folate to compensate for endothelial THBP deficiency may thus rationalize the growing number of clinical studies demonstrating that oral intakes of folate in the range of 5–10 mg daily can improve endothelium-dependent vasodilation in patients with various vascular disorders – even when their baseline homocysteine levels are in the healthful range [72,74,80,82,84]. In some of these studies, the researchers concluded that improvement in endothelial function did not correlate with reductions in homocysteine [80,83]. Perhaps the clearest proof that high-dose folate has an effect independent of homocysteine modulation is offered by a study in which oral pretreatment with folate alleviated the adverse influence of a fatty meal on endothelial function [72]. The impact of dietary folate on elevated homocysteine appears to be optimized at intakes of less than a mg daily [85] – whereas much higher intakes may be required for optimal impact on dysfunctional endothelium (albeit the dose dependency of this benefit requires further clarification).

A novel strategy – joint administration of arginine and high-dose folate

It thus appears that two of the most pathologically significant targets of oxidative stress in dysfunctional endothelium are DDAH and THBP, and that the impact of the resulting deficits of DDAH and THBP bioactivity can be compensated by supplementing with adequate intakes of arginine and folic acid, respectively. This raises the intriguing possibility that *joint administration* of arginine and high-dose folate could have a complementary and

highly beneficial impact on endothelial function in many vascular disorders. Such supplementation would not prevent excessive superoxide from quenching NO, but it might be somewhat helpful in this regard by diminishing the production or half-life of endothelial superoxide. No doubt there are other significant targets of oxidative stress that need to be addressed – activation of the pro-inflammatory transcription factor NF- κ B, for example [86–88] – but, even here, the increased endothelial production of NO achievable with arginine/folate therapy would be expected to down-regulate NF- κ B activation [89–91].

Of particular relevance to this concept is a recent study examining *ex vivo* endothelial function in hypercholesterolemic mice genetically prone to atherosclerosis [92]. Acetylcholine-triggered endothelium-dependent relaxation of aortic rings derived from these mice was subnormal in comparison to that seen with aortic rings from control mice. Adding arginine to the medium had little detectable influence on this dysfunction; adding THBP produced a modest improvement; adding arginine and THBP simultaneously virtually *normalized* endothelial function. This intriguing study thus lends support to the thesis that joint administration of arginine and high-dose folate may have particular utility in the prevention and treatment of vascular diseases characterized by endotheliopathy.

Suppressing superoxide production

Alternatively or (preferably) adjunctively, excessive endothelial superoxide production can be addressed by controlling the risk factors that boost activation of the endothelial NAD(P)H oxidase – namely, elevations of LDL cholesterol, angiotensin II, free fatty acids (associated with insulin resistance syndrome, obesity, and fatty meals), glucose, insulin (even in the context of insulin resistance), and possibly certain natriuretic factors (marinobufagenin?) evoked by salty diets [4,93–103] in addition, hypertension *per se*, via induction of cyclic strain, may activate this enzyme [104,105]. Part of the benefit of statin therapy may stem, not from controlling LDL cholesterol, but by down-regulating activation of NAD(P)H oxidase, an effect that may result from decreased isoprenylation of a G-protein crucial to the activity of this enzyme complex [106,107]; statins also have the potential to up-regulate expression of eNOS while down-regulating that of the type 1 angiotensin II receptor [108–110]. The natural waxy alcohols known as policosanols, a safer alternative to

the statins in the management of hypercholesterolemia [111,112], may have similar potential, in light of recent evidence that they down-regulate expression of HMG-CoA reductase and thus, like statins, could be expected to suppress isoprenylation of proteins. Endothelial NADPH activity can also be down-regulated by estrogen [113,114], and it is particularly fortunate that the anti-carcinogenic estrogen analog raloxifene appears to share this activity [115].

Superoxide is not the only problem

Evidently, factors other than superoxide excess can contribute to a decline in endothelial NO production. Decreased expression of eNOS is encountered in estrogen withdrawal [116–118], and in association with a sedentary lifestyle, presumably owing to lack of exercise-induced shear stress [119–126]; this enzyme is also down-regulated by the cytokine tumor necrosis factor [127–129], produced by intimal foam cells in atherosclerotic lesions. Expression of eNOS is also influenced by insulin (positively) and hyperglycemia (negatively) [130–132] – these effects might be relevant to endothelial dysfunction in insulin resistance and diabetes. Decreased endothelial membrane potential, in addition to boosting superoxide production by NAD(P)H oxidase, also impedes calcium influx, thus limiting the availability of the intracellular free calcium required for full activity of eNOS [101,133]. Subnormal insulin activity attributable to insulin resistance or insulin deficiency may prevent a post-translational phosphorylation of eNOS (at Ser 1179) that can boost enzyme activity by increasing its affinity for calcium/calmodulin while increasing its V_{max} [134–136]. Shear stress and IGF-I also stimulate this phosphorylation [137,138]; the latter effect possibly accounts for the endothelial dysfunction associated with growth hormone deficiency [139,140]. Finally, since eNOS has a slightly alkaline pH optimum, intracellular acidity can suppress eNOS activity [141–143]. It is not unlikely that at least some of these factors play a role in the relative failure of endothelial NO production that characterizes many vascular disorders – and that improvement of the vascular risk factor profile will ameliorate these factors, such as eNOS activity increases.

It will be important to determine to what extent these factors – as well as increased superoxide production – are inherent to endothelial aging; clearly, the aging process impairs endothelial NO activity, even in subjects who remain healthy and

have moderate risk factors [144–148]. Studies with cultured endothelial cells conclude that senescence is associated with a marked reduction in the expression of eNOS [149,150], whereas clinical and animal studies suggest that increased endothelial superoxide production is inherent to the aging process [146,151–153]. The intriguing possibility that lifestyle regimens which optimize endothelial NO activity may literally slow the rate of endothelial aging, is discussed in a separate communication [154].

It is clear that endothelial function is extremely complex, and that factors other than superoxide excess can and do impair NO bioactivity. Nonetheless, it is equally clear that overproduction of superoxide plays a central role in mediating the endotheliopathy encountered in a wide range of vascular disorders. The fact that DDAH and THBP are key targets of this superoxide, helps to rationalize the frequently favorable impact of arginine and of high-dose folate on dysfunctional endothelium, and suggests that it would be worthwhile to examine the clinical utility of joint administration of arginine and high-dose folate. Such supplementation should not be expected to completely normalize the function of oxidatively stressed endothelium, but it might prove to be a very useful component of more complex therapeutic strategies addressing endothelial dysfunction.

One technical issue should be addressed briefly. At present, the US FDA limits the folic acid content of non-prescription supplements to 400 mcg (and 800 mcg in prenatal formulas), owing to concerns that, in people who are developing pernicious anemia, concurrent ingestion of high doses of folate may prevent the anemia that typically is the first symptomatic manifestation of this disorder. As a result, an appropriate diagnosis may be delayed, increasing the likelihood that the syndrome will be diagnosed and treated only after potentially irreversible neurological damage has occurred [155]. The dearth of published case histories documenting this outcome in folate-supplemented subjects suggests that the FDA's concern in this regard is disproportionate to the actual risk [156]; moreover, the suspicion that supplemental folate may accelerate progression of neuropathy in pernicious anemia, appears to be groundless [157]. Nonetheless, it is feasible to completely eliminate such risk by including a sufficient amount of vitamin B12 — e.g., 400 mcg per daily dose — in folate supplements [156]. Even in the absence of intrinsic factor, about 1% of dietary B12 achieves absorption by diffusion [158–160]; thus, a sufficient oral intake of B12 can prevent and correct B12 deficiency, even when pernicious anemia severely limits gastric produc-

tion of intrinsic factor. Clearly, there should be no rational objection to high-dose folate supplements that concurrently provide an ample amount of vitamin B12.

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