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High-dose folate may improve platelet function in acute coronary syndrome and other pathologies associated with increased platelet oxidative stress

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Summary Although nitric oxide of endothelial origin plays a major role in warding off inappropriate thrombus formation, platelets also express the ''constitutive'' isoform of nitric oxide synthase (cNOS). Activation of this enzyme by calcium influx during platelet aggregation provides an important feedback signal that dampens platelet recruitment. Platelets also express a membrane-bound NAD(P)H oxidase complex, activated by collagen receptors, that produces superoxide. Superoxide can directly quench NO; moreover, by giving rise to peroxynitrite, it can oxidize the cNOS cofactor tetrahydrobiopterin (BH4), thereby suppressing cNOS activity and converting it to superoxide generator. In a canine model of acute coronary syndrome, infusion of BH4 has been shown to prevent thrombus formation. Platelets from patients with acute coronary syndrome produce markedly less NO than do control platelets. A reasonable explanation for these findings is that episodic contact with collagen boosts platelet superoxide production, oxidizing BH4. Since 5-methyltetrahydrofolate can reduce oxidized BH4, or otherwise compensate for its deficiency, supplementation with its precursor folic acid may improve platelet function in acute coronary syndrome and possibly reduce risk for coronary thrombosis in other at-risk patients. Other research demonstrates that superoxide production is increased, and nitric oxide production diminished, in platelets of diabetics; the ability of glutathione — a peroxynitrite scavenger — to largely ameliorate these abnormalities, is consistent with a prominent role for BH4 deficiency in diabetic platelet malfunction. Reports that platelet NO production is decreased, and/or superoxide production increased, in patients with disorders associated with insulin resistance syndrome, suggest that BH4 deficiency — potentially remediable with high-dose folate — may likewise contribute to the platelet hyperreactivity noted in these disorders. Supplemental vitamin C and arginine also have the potential to boost platelet production of NO Increased intakes of taurine, magnesium, gamma-tocopherol, fish oil, and garlic may help to stabilize platelets by additional mechanisms. As a complement to the proven benefits of low-dose aspirin, a supplemental regimen emphasizing these nutrients in appropriate doses may act directly on platelets to further diminish risk for thrombotic episodes.

Platelet-derived NO serves as a feedback signal during aggregation

Although endothelial-derived nitric oxide (NO) — as well as prostacyclin — plays a key physiological role

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in preventing inappropriate platelet aggregation near intact endothelium, platelets also express the so-called constitutive (endothelial) nitric oxide synthase (cNOS) [1—4]. This enzyme is activated by various stimuli that promote aggregation, presumably owing to the increase in free intracellular calcium which such agonists evoke; the resulting increase in NO generation acts as a feedback signal that dampens platelet aggregation and — more substantially — platelet recruitment. Studies with cNOS inhibitors, as well as with cNOS knock-out mice, confirm that this feedback signal is of physiological importance for preventing excessive thrombus formation [4—6]. Insulin acts on platelets to up-regulate their cNOS activity [7—9], apparently via phosphorylation mediated by cAMP-activated kinase (AMPK) rather than Akt [10]. (Insulin-mediated activation of AMPK is a distinctive feature of platelets.) The ability of insulin to stimulate platelet NO production is blunted in insulin resistance syndrome, contributing to prothrombotic tendencies in this disorder [9,11,12].

Platelets express soluble guanylate cyclase [13,14]; NO generated by both platelets and healthy endothelium activates this enzyme, boosting platelet levels of cGMP. By interacting with the cGMP-inhibited cAMP phosphodiesterase, the most prominent isoform of cAMP phosphodiesterase expressed in platelets [15,16], an increase in cGMP also boosts platelet cAMP levels, and potentiates the increase in cAMP induced by prostacyclin and other agonists that stimulate adenyl cyclase [17—19]. Both cGMP and cAMP act by a variety of complementary mechanisms to dampen and curtail the increase in free intracellular calcium evoked by pro-aggregant agonists, thus promoting platelet stability [9,20].

Platelet superoxide promotes oxidation of tetrahydrobiopterin

Platelets also have the capacity to generate superoxide, a functional antagonist of NO [21]. As is well known, superoxide interacts directly with NO to ''quench'' it, yielding the highly active oxidant peroxynitrite [22]. The latter can compromise NO synthase activity by oxidizing tetrahydrobiopterin (BH4), an obligate cofactor for this enzyme [23,24]. Deficiency of BH4 not only diminishes the ability of NOS to generate NO, but also converts this enzyme to a generator of superoxide [25—28]. Other major sources of superoxide in platelets include the membrane-bound NAD(P)H oxidase [29—31] — similar or identical to that found in phagocytes, endothelial, and smooth muscle cells — and lipoxygenase activity [32]. Platelet mitochondria and xanthine oxidase may also contribute to platelet generation of superoxide [33,34].

Certain pro-aggregant agonists — most notably collagen, which comes into contact with platelets when the endothelial barrier is breached — activate platelet NAD(P)H oxidase [31]. The resulting production of superoxide up-regulates aggregation and, more substantially, subsequent platelet recruitment, by antagonizing NO bioactivity [31]. Conversely, measures which inhibit this enzyme complex have an anti-aggregant effect — though not in mice genetically deficient in cNOS [35,36]. Potentially, circumstances which stimulate platelet superoxide production could induce BH4 deficiency, severely compromising the protective activity of platelet cNOS. Indeed, there is reason to believe that this phenomenon is associated with, and likely exacerbates, acute coronary syndrome, in which platelets interact with the sub-endothelial intimal collagen of damaged arteries.

Platelet BH4 deficiency in acute coronary syndrome

This conclusion is prompted by a study which evaluated the impact of parenteral BH4 administration on platelet aggregation in a canine model that simulates clinical acute coronary syndrome [37]. In this model, constriction of a coronary artery accompanied by endothelial damage at the point of constriction promotes the transient production of thrombi, which can be quantified by assessing temporary cessations of flow through the stenosed artery (''cyclic flow variations'') [38]. In dogs who developed these cyclic flow variations following stenosing surgery, intravenous administration of BH4 (10—30 mg/kg) was found to dose-dependently diminish the frequency of these variations — a benefit that was lost if the NOS inhibitor NMMA was co-infused. *Ex vivo* platelet aggregation was also studied during this study; the pro-aggregant response to ADP increased substantially during the cyclic flow variations, but normalized after administration of BH4. The nitrotyrosine content of platelets — indicative of peroxynitrite formation — also increased during cyclic flow variations, but was diminished after BH4 infusion. The researchers also measured platelet BH4 levels; these decreased by more than 50% during cyclic flow variations, but achieved supra-normal levels after BH4 was administered.

These findings are nicely concordant with those of a study which assessed *ex vivo* NO production

by platelets obtained from patients who had either stable or unstable angina; platelets obtained from patients with acute coronary syndrome generated only one-seventh as much NO as those obtained from patients with stable angina [39]. Unfortunately, this study did not evaluate the role of BH4 in this phenomenon.

Platelet-stabilizing potential of high-dose folate

While acute parenteral administration of BH4 can be envisioned in coronary emergencies, BH4 is probably too oxidant labile to be suitable for oral administration [27,40–42]. However, recent studies show that 5-methyltetrahydrofolate – the chief metabolite of the vitamin folic acid which circulates in plasma – can restore eNOS activity when this has been diminished by reversible oxidation of BH4 [43,44]. The mechanism of this effect is still somewhat unclear – some data suggest that 5-methyltetrahydrofolate literally ''pinch-hits'' for THBP by occupying its binding site on eNOS, while other findings indicate that 5-methyltetrahydrofolate regenerates THBP from its oxidized dihydro-derivative by a reduction reaction [45–47]. In any case, intra venous administration of 5-methyltetrahydrofolate, as well as oral administration of folic acid in daily doses of 5–10 mg (far higher than is required to control homocysteine), have been shown to have a favorable impact on NO-mediated endothelium-dependent vasodilation in patients afflicted with various endotheliopathies characterized by increased endothelial superoxide generation [40,43,48–55]; it is clear that this phenomenon is not simply a function of homocysteine control [40,51]. In light of the foregoing discussion, it is reasonable to speculate that these measures would likewise exert a direct anti-aggregant effect on platelets in acute coronary syndrome and possibly other clinical syndromes in which excessive platelet surperoxide production precipitates significant oxidation of platelet BH4.

If this thesis is correct, it should be possible to demonstrate that some sufficient oral intake of folic acid has a favorable effect on *ex vivo* platelet function in acute coronary syndrome. Not unlikely, such supplementation would also dampen thrombotic tendencies *in vivo* by promoting improved endothelial NO bioactivity. It is also reasonable to envision that, in patients with stable coronary disease, such supplementation might have a preventive effect, helping to preserve platelet (and endothelial) BH4 levels if and when endothelial trauma threatens to precipitate unstable angina or myocardial infarction. Nonetheless, it should be noted that these predictions are contingent on the ability of 5-methyltetrahydrofolate to indeed boost BH4 levels in oxidant-stressed platelets – a proposition that remains to be proved.

BH4 deficiency as a mediator of diabetic platelet dysfunction

In addition to acute coronary syndrome, platelet capacity to generate NO is reported to be diminished in various chronic conditions associated with insulin resistance and endotheliopathy – such as diabetes, hypertension, tobacco addiction, and non-diabetic insulin resistance syndrome [9,56,56–60]. Conversely, excessive platelet production of superoxide has been observed in most of these disorders, as well as hypercholesterolemia [57,60–62]. It is therefore reasonable to inquire whether platelet BH4 deficiency might contribute to the platelet hyperreactivity observed in these syndromes.

Graier and colleagues have reported that, in the platelets of type 2 diabetics, superoxide production is enhanced, whereas NOS activity is substantially decreased; as compared to platelets derived from healthy controls, basal NO production by diabetic platelets was 85% lower, and thapsigargin-stimulated production was 64% lower [57]. Intriguingly, pre-incubation with 10 mmol glutathione markedly suppressed platelet superoxide production in diabetics – but not controls – and nearly normalized the up-regulated calcium signaling in diabetic platelets. Conversely, induction of glutathione deficiency in normal platelets boosted their production of superoxide and increased their calcium signaling. This observation is hard to square with the fact that glutathione is not a superoxide scavenger. However, glutathione *does* function to quench peroxynitrite, acting either directly or via glutathione peroxidase [63–68]. If diabetic platelets generate increased amounts of peroxynitrite, increased glutathione availability would be expected to protect BH4, improving the ability of cNOS to produce NO while suppressing superoxide production by this enzyme. Indeed, Mazzanti and Mutus and not only confirm that NO production is notably reduced in diabetic platelets, but also note that NOS-dependent superoxide production is increased 20-fold in these platelets [69]. The authors further state that ''the diabetic platelet that produces less GSH cannot appropriately quench the overproduction of peroxynitrite.

As a consequence, platelet proteins would bear the full brunt of the oxidative potential of peroxynitrite, resulting in platelet damage and dysfunction''. Remarkably, these observations were made 2 years before the oxidizing impact of peroxynitrite on BH4 was demonstrated. In a more recent study, parenterally administered glutathione in type 2 diabetics was shown to increase the cNOS activity of their platelets [70]. Since platelet glutathione levels may be subnormal in type 2 diabetics [71,72], the peroxynitrite generated in these platelets may be especially detrimental to cNOS activity.

Arguably, episodic increases in platelet superoxide production — by whatever mechanism — could induce a platelet BH4 deficiency that converts cNOS to a superoxide generator; thus, cNOS might maintain superoxide production — and BH4 oxidation — even after the initiating burst of superoxide production remits. There is evidence that hyperglycemia per se can increase superoxide production in platelets, possibly through mitochondrial mechanisms [33]. These considerations suggest that BH4 deficiency may be a key mediator of diabetic platelet dysfunction. Evidently, this could be tested by examining the impact of pre-incubation with BH4 or 5-methyltetrahydrofolate on platelet production of superoxide and NO, and on aberrant platelet calcium signaling. If this study had the expected outcome, it would then be appropriate to determine whether sufficiently high intakes of folic acid could normalize or at least improve *ex vivo* platelet function in diabetics.

Is platelet BH4 diminished in insulin resistance syndrome?

What about non-diabetic insulin resistance syndrome? It is known that the ability of insulin to stabilize platelets — attributable to activation of cNOS — is diminished in this syndrome [9]. This could reflect a dysfunction in the signaling mechanism whereby insulin evokes an activating phosphorylation of cNOS. On the other hand, diminished platelet BH4 levels could be at least partially responsible for this phenomenon. Evidently, this issue could be addressed by examining the impact of BH4 or 5-methyltetrahydrofolate on insulin-stimulated NO production in platelets from insulin-resistant subjects. It should be noted that platelets from obese insulin-resistant subjects have been found to be relatively resistant to the anti-aggregant effects of nitric oxide; although basal guanylate cyclase activity appears to be normal in these platelets, the responsiveness of this enzyme to NO is decreased, for reasons that remain unclear [9,73]. Hence, boosting the BH4 content of these platelets could not be expected to fully reconstitute normal platelet function.

Arguably, subjects with acute coronary syndrome, diabetes, or insulin resistance syndrome (including hypertension — likewise associated with platelet dysfunction) should in any case be treated with high-dose folate, as these conditions are associated with superoxide-associated endotheliopathy likely to be benefited by this strategy [43,44]. But the impact of such supplementation on *ex vivo* platelet function also merits study — in part because the dose-dependency of folate's impact on endothelium might not be identical to that of its impact on platelets.

If high-dose folate can indeed improve platelet function in various endotheliopathies, it should be noted that the effect of such therapy would be to restore normal platelet function. In other words, as contrasted with pharmaceutical measures which inhibit platelet signaling mechanisms, high-dose folate would not be expected to increase risk for hemorrhagic complications.

Additional nutritional measures can stabilize platelets

Vitamin C also has the ability to reduce oxidized BH4 to its active form [41,42]. This is thought to explain why ample oral intakes of vitamin C can improve endothelium-dependent vasodilation in subjects afflicted with a wide range of endotheliopathies [74—82]. The scope of this benefit may be limited, however, by the fact that endothelial uptake of ascorbate is maximized at a plasma concentration of 100 μM [42,83], said to be achieved by daily intakes as low as 500 mg [74]. (Higher plasma concentrations of ascorbate, achieved by intravenous administration, or transiently after high oral doses, have the potential to influence endothelial function by quenching extracellular superoxide.) Presumably, improved vitamin C status has the potential to directly influence platelet function when platelet BH4 is challenged by oxidant stress. Indeed, there are a number of reports that ascorbate administered orally, intravenously, or in vitro can reduce platelet aggregation [84—89,60,90]. One of these reports assessing the impact of a single oral dose of 2 g in chronic smokers and non-smokers, is of exceptional interest; in the smokers, the vitamin C suppressed aggregation, increased NO production, and decreased nitrotyrosine content — normalizing these parameters with

respect to those seen in the non-smokers [60]. These results are all consistent with remediation of an uncoupled cNOS owing to normalization of BH4 levels. Analogously, vitamin C has been shown to increase NOS activity in neutrophils [91] — an effect which boosts the platelet-stabilizing activity of these cells *in vivo* [92].

Surprisingly, high-dose supplementation with arginine, the substrate for eNOS, can reduce platelet aggregability not only in patients with endotheliopathy [93] — associated with elevated levels of the competitive inhibitor ADMA [94] — but in normal subjects as well, even though supplemental arginine does not influence endothelial function in healthy subjects [95]. A similar effect has been reported when arginine is infused intravenously in healthy subjects [96]. In vitro, platelet cGMP levels rise progressively as medium arginine concentrations are increased through the range 100—500 μM [97]; normal plasma arginine levels are near the bottom of this range. This effect is delayed in onset, but can be accelerated by the cell-permeabilizing agent saponin — suggesting that platelet uptake of arginine is a limiting factor. Thus, the arginine responsiveness of platelets may reflect an intracellular concentration that is far lower than that of endothelial cells and near the Km of cNOS for arginine, owing to limited capacity for arginine transport. Indeed, the intraplatelet arginine level is said to be only about 1 μM [97] — though no source is cited for this datum. In any case, it is reasonable to anticipate that supplementary arginine could complement the BH4-boosting activities of folate and vitamin C in promoting the anti-aggregant efficacy of platelet-derived NO.

Other nutritional measures with the potential to down-regulate platelet reactivity include supplementation with taurine [98—101], magnesium [102,103], gamma-tocopherol [104], and long-chain omega-3 fatty acids,[105] as well as ingestion of garlic [106—109]. Each of these measures appears to be complementary to the anti-aggregant impact of low-dose aspirin, which targets thromboxane production. Although fish oil likewise suppresses thromboxane production — albeit less effectively than aspirin — it potentiates the impact of aspirin on bleeding times, for reasons that remain unclear [110,111]. In light of the dramatic impact on cardiovascular mortality and morbidity achievable with low-dose aspirin [112], it is reasonable to anticipate that complementary nutritional measures which likewise can stabilize platelets may have a very worthwhile impact in this regard. Moreover, the nutrients cited here — including high-dose folate — likely can work in other ways to promote vascular health, and thus may be recommended as key components of a preventive health program.

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