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Editorial

Dihydrofolate reductase and biopterin recycling in cardiovascular disease

Nitric oxide, generated by the nitric oxide synthase (NOS) enzymes, plays pivotal roles in cardiovascular homeostasis and in the pathogenesis of cardiovascular disease. The NOS cofactor, tetrahydrobiopterin (BH4), is an important regulator of NOS function, since BH4 is required to maintain enzymatic coupling of L-arginine oxidation, to produce NO. Loss or reduction of BH4 is associated with NOS uncoupling, resulting in production of superoxide rather than NO. Electron paramagnetic resonance spectroscopy studies have shown that BH4 both stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain, as the initiating step of L-arginine oxidation. In this reaction BH4 forms the protonated trihydrobiopterin cation radical, and is then reduced by electron transfer from NOS flavins [1]. When BH4 availability is limiting, electron transfer from NOS flavins becomes uncoupled from L-arginine oxidation, the ferrous-dioxygen complex dissociates, and superoxide is produced from the oxygenase domain [2].

Recent evidence suggests that BH4 has important roles in cardiovascular disease states such as diabetes [3,4], atherosclerosis, pulmonary hypertension [5] and ischemia-reperfusion injury [6]. These pathophysiologic conditions are associated with reduced BH4 levels and NOS uncoupling that contributes to oxidative stress.

BH4 biosynthesis is regulated by the rate-limiting enzyme, guanosine triphosphate cyclohydrolase I (GTPCH), that catalyzes the formation of dihydroneopterin triphosphate from GTP, and BH4 is generated by further steps catalyzed by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase. Under steady-state conditions, GTPCH expression and activity are directly related to cellular BH4 levels [7]. Experimental studies have shown that increasing BH4 biosynthesis, for example in transgenic models, leads to increased BH4 levels and can increase or restore NOS coupling in cardiovascular disease states [3]. However, low BH4 levels in pathophysiologic conditions appear to be associated with increased BH4 oxidation as evidenced by marked accumulation of BH2, rather than changes in BH4 biosynthesis per se [3,8]. Although BH4 is susceptible to oxidation by many reactive oxygen species (ROS) including superoxide [9], due to the near-diffusion rate of the reaction between superoxide and NO, peroxynitrite ($ONOO^-$) is believed to be primarily responsible for oxidation of BH4 to BH2 in vivo [10,11]. Induction of BH4 oxidation leads to NOS uncoupling, an effect that can be prevented, in part, by antioxidants such as ascorbic acid [12]. BH4 oxidation leads to formation of other pterin species that are either totally inactive as NOS cofactors, such as xanthopterins [6], or may lead to formation of the partially oxidized 7,8-dihydrobiopterin (BH2), that can compete with BH4 for NOS binding, but cannot support NOS cofactor activity. Recent studies reveal that BH4 and BH2 bind eNOS with equal affinity and that BH2 can efficiently replace eNOS-bound BH4, resulting in eNOS uncoupling [13]. Thus, the relative abundance of BH4 vs. BH2, rather than the absolute concentrations of BH4 alone, appears to be a key determinant of NOS uncoupling [14], so regulation of BH4 vs. BH2 levels may be as important in cardiovascular disease pathogenesis as BH4 biosynthesis. Indeed, in clinical studies pharmacological supplementation of BH4 improves endothelium-dependent relaxations and augments NO-mediated effects on forearm blood flow in smokers and those with diabetes and elevated cholesterol [15–17]. However, this may be due to nonspecific scavenging of superoxide by high dose BH4 treatment. Furthermore, BH4 might be subject to oxidation in cardiovascular disease states that would render it ineffective. The mechanisms that regulate BH4 availability *in vivo* are not fully understood, and approaches to develop BH4 as a therapeutic target remain speculative unless a better understanding of BH4 biosynthesis and the role of BH4 recycling within the cardiovascular system can be gained.

Dihydrofolate reductase (DHFR; E.C 1.5.1.3) plays a central role in folate metabolism, maintaining cellular pools of tetrahydrofolate by reduction of dihydrofolate. In addition to these key roles in folate metabolism, DHFR can reduce BH2, thus regenerating BH4 as part of the "pterin salvage pathway" [18]. However, the role of this pathway and the extent to which it regulates intracellular BH4 levels in vivo remains unclear, with recent evidence suggesting that this "BH4 recycling" function of DHFR may have particular relevance to NOS coupling in cardiovascular disease. Chalupsky and Cai [19] previously reported that DHFR is required to maintain BH4 levels and eNOS coupling in endothelial cells exposed to angiotensin II (Ang II), that is known to increase endothelial cell production of reactive oxygen species. The importance of DHFR, identified by siRNA knockdown or inhibition by methotrexate, appears to be more prominent at low levels of GTPCH expression, where reduced cellular BH4 increases the susceptibility to eNOS uncoupling, including "self-induced" eNOS uncoupling resulting from uncoupled eNOS-dependent BH4 oxidation [20], and conditions where BH2 levels are high [21].

Some previous studies have shown that changes in DHFR levels or activity are diminished in experimental models of cardiovascular disease states, suggesting that insufficient recycling of BH2 to BH4 by DHFR is at least in part responsible for the reduced BH4 levels and the accumulation of BH2, leading to eNOS uncoupling. For example, DHFR protein levels are significantly decreased in streptozotocin-induced diabetic mice and diabetes-induced impairment of cardiac myocyte function is exacerbated following treatment of the mice with the DHFR inhibitor, methotrexate [22]. Furthermore, reduced DHFR activity in adult cardiac myocytes underlies their limited capacity to synthesize BH4 after cytokine stimulation following treatment of rat cardiac allograft recipients with sepiapterin [23]. Insufficient DHFR activity might also explain impaired vasorelaxation in atherosclerotic vessels from hypercholesterolemic rabbits, despite exposure to sepiapterin, that increases biopterin levels through BH2, requiring DHFR to increase BH4 [24].

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In this issue of the *Journal of Molecular and Cellular Cardiology*, Gao et al. expand the hypothesis that DHFR and the salvage pathway play an important role in the regulation of intracellular BH4 levels, by protecting endothelial cells from the effects of Ang II-induced oxidative stress [25]. Using a novel HPLC based assay for the measurement of DHFR activity in cell lysates and tissues, it was determined that incubation with Ang II reduced NO and BH4 bioavailability, due to reduced DHFR activity. However, pre-incubation with folic acid prevented these deleterious effects of Ang II, but folic-acid mediated protection was lost in cells following either DHFR knockdown with siRNA, or DHFR inhibition with methotrexate. *In vivo*, mice infused with Ang II had markedly increased aortic superoxide production and reduced aortic NO bioavailability, that was accompanied by endothelial down-regulation of DHFR. Oral administration of folic acid was effective in significantly improving NO production, while reducing superoxide levels. Folic acid treatment also prevented the Ang II-induced reduction in endothelial DHFR protein levels without any significant effects on either aortic GTPCH activity or MTHF levels.

Taken together, these data suggest that DHFR may have an important role in regulating endothelial cell BH4 and NO bioavailability under conditions of oxidative stress. Although the studies of Gao et al. highlight a potential role for DHFR in BH4 homeostasis, it remains possible that the effects of DHFR on endothelial function could be due to the important regulatory role of DHFR on folate metabolism. Several studies have shown that folate supplementation can improve endothelial function in subjects with hyperhomocysteinemia, hypercholesterolemia [26,27], diabetes [28], coronary artery disease [29], and in smokers. At least some of the effects of folate supplementation in human atherosclerosis appear to be due to prevention of peroxynitrite-mediated BH_4 oxidation, and improving eNOS coupling [30,31]. However, an effect of folate on DHFR was not considered in these previous studies, so some of the salutary effects of folates on endothelial function, BH4 and eNOS coupling might be explained by the novel action of folic acid on DHFR activity proposed by Gao et al. Future studies are required to fully understand the regulation of endothelial function by folate and BH4, and the role of DHFR vs. direct effects of folates.

Regulation of net BH4 bioavailability within the endothelium is likely to result from a complex equilibrium between *de novo* synthesis, oxidative loss, recycling pathways and biopterin transport between the extracellular and intracellular compartments. BH4 oxidation and recycling, and the regulation of the relative abundance of BH4 and BH2 by DHFR and other mechanisms, are new aspects of NOS pathophysiology with therapeutic implications.

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