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Reduced vascular tetrahydrobiopterin (BH_4) and endothelial function with ageing: is it time for a chronic BH_4 supplementation trial in middle-aged and older adults?

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Ageing is associated with a reduction in endothelium-dependent dilatation (EDD) of both resistance and conduit arteries in humans and rodents (Eskurza *et al.* 2005; Delp *et al.* 2008). This is partly the result of a reduction in endothelial-derived nitric oxide (NO) bioavailability. The decrease in NO bioavailability probably is mediated by several possible mechanisms including a decrease in synthesis of NO by the enzyme endothelial NO synthase (NOS), increased degradation of NO by reactive oxygen species such as superoxide anion ($O_2\cdot^-$), or a combination of both. A decrease in synthesis of NO is not likely to be mediated by a decrease in NOS protein expression or activity as there is no consistent evidence for such changes with ageing, and indeed, the opposite has been reported. Rather it is more likely that impaired NO synthesis is explained by enhanced oxidation or decreased synthesis of endothelial tetrahydrobiopterin (BH_4), a key cofactor for the NOS enzymes that is obligatory for NO synthesis.

BH_4 is a naturally occurring enzyme cofactor which is synthesized *de novo* from guanosine 5′-triphosphate (GTP) and is controlled by the rate limiting enzyme GTP cyclohydrolase I (GTPCH I). Conversely, BH_4 can also be generated via a 'salvage pathway' by the enzymes sepiapterin reductase and dihydrofolate reductase; however, the salvage pathway cannot compensate for a reduction in GTPCH I-mediated *de novo* synthesis of BH_4. Interestingly, BH_4 is a powerful reducing agent and is highly susceptible to oxidation by reactive oxygen species such as peroxynitrite. $O_2\cdot^-$ produced from NADPH oxidase and/or other enzymatic sources (xanthine oxidase, mitochondria, uncoupled NOS) reacts with NO and forms peroxynitrite before being inactivated by the enzymatic antioxidant superoxide dismutase. Because vascular oxidative stress develops with ageing, it is plausible that BH_4 becomes oxidized leading to uncoupling of endothelial NOS with subsequent increased production of $O_2\cdot^-$. However, direct experimental evidence to support this hypothesis is still lacking.

A reduction in L-arginine as a result of increased degradation by the enzyme arginase I has been advanced as an alternative mechanism of endothelial NOS uncoupling and reduced NO-mediated EDD with ageing (Santhanam *et al.* 2007). Because the K_m value of arginase I is significantly higher than that of NOS enzymes, under normal physiological conditions arginase I and NOS should not be in competition for L-arginine. However, Berkowitz and colleagues recently extended previous findings of the role of increased arginase I activity in the age-related impairment of EDD by demonstrating that arginase I is post-translationally modified in aged vessels by *S*-nitrosylation of two cysteine residues (Santhanam *et al.* 2007). They reported that NO produced from inducible NOS nitrosylates arginase I decreasing its K_m value ~6-fold, therefore increasing its affinity for L-arginine and supporting the idea that a reduction in L-arginine secondary to an increase in arginase I activity contributes to impaired NO bioavailability and EDD of vessels of aged rodents.

In a recent study in *The Journal of Physiology*, Delp *et al.* (2008) addressed several important questions related to the contribution of L-arginine and BH_4 bioavailability to impaired flow-induced EDD of the skeletal muscle microcirculation with vascular ageing. They first asked if an increase in arginase I activity in resistance arteries (soleus 1A arterioles) of aged rats leads to a decrease in EDD as reported in large conduit (aorta) arteries (Santhanam *et al.* 2007). Delp and colleagues demonstrated that *ex vivo* administration of the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine or administration of exogenous L-arginine to the bath did not improve EDD of arterioles in the older rodents. Consistent with these observations, they found no difference in L-arginine content in the arterioles between old and young rats. These findings indicate that a reduction in L-arginine as a result of either increased arginase I activity or some other mechanism does not contribute to impaired EDD of 1A arterioles in older rats. The reason for the divergent results between these studies is unclear, but it is possible that they reflect differences in regulation of vascular L-arginine between conduit and resistance arteries with ageing in rodents.

Delp and colleagues next asked if the decrease in EDD is a result of a reduction in vascular BH_4 content. Indeed, Delp *et al.* reported that total BH_4 content of soleus 1A arterioles was reduced ~60% in the older compared with the young rats. This is in contrast to findings reporting that BH_4 content was not reduced in aortas of older compared with young mice (Blackwell *et al.* 2004). The explanation for these conflicting results is not clear, but differences in the arterial segments investigated (aorta *versus* arterioles) and/or species differences (rats *versus* mice) may be involved. Delp and colleagues also demonstrated that *ex vivo* administration of sepiapterin, a precursor for BH_4, in isolated soleus 1A arterioles resulted in a 52% improvement in flow-induced EDD in old compared with young rats, although the age-related reduction in EDD was not restored to levels of the young animals. Moreover, improvement in EDD was abolished by administration of the NOS inhibitor N^{ω}-monomethyl-L-arginine so that EDD was not different in old compared with young rats in the absence of NO production. These observations suggest that the ~50% improvement in EDD from sepiapterin administration (i.e. BH_4) in the older rats was NO mediated, but that additional mechanisms may be involved.

In humans, Eskurza *et al.* (2005) demonstrated that a single high dose of oral BH_4 restored EDD of the brachial conduit artery in sedentary older adults to that of young adults. That BH_4 administration fully restored EDD in older humans, but not in the study by Delp and colleagues obviously could have been explained by inherent differences between humans and rodents. However, because intracellular levels of BH_4 were not measured in the arterioles after administration of sepiapterin in the study by Delp *et al.* it is unknown if sepiapterin

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restored the vascular BH_4 levels in the older rodents. Use of sepiapterin (as opposed to BH_4) to increase intracellular BH_4 levels depends on the activity of the salvage pathway enzymes sepiapterin reductase and dihydrofolate reductase. It is possible that the intracellular BH_4 synthesis was limited in the older animals by reduced content and/or activities of these enzymes. Nonetheless, Delp and colleagues provide the first evidence that BH_4 content (and not L-arginine) is reduced in vascular tissue of skeletal muscle arterioles with ageing in rodents and that exogenous administration of a BH_4 precursor partially restores the age-related impairment of EDD.

Several important questions remain to be answered concerning the role of BH_4 and endothelial dysfunction with ageing. Is the decrease in BH_4 with ageing mediated by increased oxidation of BH_4 to BH_2 and other biopterins, or is BH_4 synthesis decreased (e.g. via a reduction in GTPCH I activity). Delp and colleagues measured only total BH_4 and not the ratio of oxidized to reduced biopterins, and did not measure GTPCH I content or activity. Therefore, the mechanism for the age-related reduction in vascular BH_4 remains to be determined. Next, is the decrease in vascular BH_4 with ageing associated with reductions in BH_4 content of endothelial cells, smooth muscle cells, or both? Delp and colleagues studied whole arterioles and therefore it is unclear which vascular cell type is responsible for the observed reduction in BH_4. Lastly, is BH_4 (or sepiapterin) administration acting as an antioxidant or restoring intracellular BH_4 levels and 'recoupling' endothelial NOS? Some insight into this question was provided by Heitzer *et al.* (2000), who administered tetrahydroneopterin (NH_4), a compound with similar antioxidant properties as BH_4 but without the influence of endothelial NOS coupling, into the brachial arteries of chronic smokers. They demonstrated that in contrast to augmentation of impaired forearm EDD by brachial artery infusion of BH_4, NH_4 had no effect on EDD. Furthermore, results from studies using electron paramagnetic resonance spin suggest that $O_2{\cdot}^-$ scavenging by BH_4 is not a major reaction *in vivo* (Vasquez-Vivar *et al.* 2003). Thus, these findings support the idea that administration of BH_4 improves EDD by recoupling endothelial NOS and not by antioxidant actions.

Perhaps most importantly, a final question to be answered is whether BH_4 content and GTPCH I content/activity are reduced in conduit and/or resistance arteries with ageing in humans. However, although endothelial cell sampling techniques in humans are available, this will still be technically challenging because primary culturing of endothelial cells from humans is difficult and basal BH_4 and GTPCH I protein expression are barely detectable in cultured endothelial cells.

In conclusion, the study by Delp *et al.* (2008) provides further support for the potentially important role of reduced BH_4 in mediating the age-associated impairments in EDD. However, regardless of whether endothelial BH_4 content is reduced with ageing or the mechanism by which acute exogenous BH_4 administration improves EDD, it may be time to determine the efficacy of chronic BH_4 supplementation for restoring vascular endothelial dysfunction in middle-aged and older adults.

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