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Microvascular function and ageing: L-arginine, tetrahydrobiopterin and the search for the fountain of vascular youth

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Cardiovascular disease remains the most salient killer in the United States and ageing is a primary risk factor. With advancing age, there is pervasive macrovascular and microvascular dysfunction that manifests as stiffening of central elastic arteries, thickening of intimal–medial layers of the vascular wall, and diminished peripheral conduit artery/resistance artery vasodilatory capacity. Reduced endothelium-dependent vasodilatation has been shown in several prospective studies to be an independent predictor of adverse cardiovascular events.

In the healthy vasculature, nitric oxide (NO) is released from endothelial cells in response to laminar shear stress and causes vasodilatation. In addition to its vasodilatory properties, NO is anti-atherogenic: it inhibits platelet aggregation/adhesion, smooth muscle cell proliferation and lipid oxidation. Depletion of NO has been linked to several pathologies including atherosclerosis and hypertension. Understanding the mechanistic aspects of NO bioavailability, and loss thereof with ageing, has significant clinical relevance.

Synthesizing NO requires a precise admixture of substrate and cofactors. Unidirectional laminar shear stress activates endothelial nitric oxide synthase (eNOS) via phosphorylation. L-Arginine is hydroxylated to *N*-hydroxy-L-arginine and then further oxidized to NO and L-citrulline. NO diffuses into smooth muscle cells, activates guanylate cyclase and induces cyclic GMP-mediated smooth muscle relaxation. Other substrate and cofactors required for this reaction include oxygen, NADPH, flavin, heme and tetrahydrobiopterin (BH4). Altering any one of these variables with ageing may set the reaction awry and attenuate vasodilatation. In an article recently published in *The Journal of Physiology,* Delp and associates examined several potential mechanisms that may contribute to reduced microvascular vasodilatory capacity with ageing (Delp *et al.* 2008).

Delp *et al.* harvested arterioles from the soleus muscles of young and old rats and exposed them to graded increases in intraluminal flow in the absence of changes in intraluminal pressure. Results revealed that arterioles from old rats exhibited a 52% reduction in flow-mediated dilatation (FMD) compared to arterioles from young rats. Vessel dilatation was also assessed following administration of the NO donor sodium nitropruside (SNP). There were no group differences in SNP-mediated dilatation suggesting similar endothelial-independent vasodilatation in arterioles from young and old rats. Confirming that the age-associated difference in microvascular vasodilatation was endothelial dependent, Delp and associates proceeded to systematically and eloquently rule out several potential limiting factors regulating the NO signalling pathway.

(a) Reduced NO bioavailability. FMD of harvested arterioles was determined following NOS blockade with N^G-nitro-L-arginine methyl ester (L-NAME). Results revealed L-NAME administration significantly reduced FMD in both groups and eliminated age-associated differences, confirming that impaired FMD with ageing is endothelial dependent and related to eNOS signalling.

(b) Reduced L-arginine supply. Reduced substrate may be rate-limiting and reduce NO formation and subsequently FMD. To examine this hypothesis, FMD of harvested arterioles was determined following administration of exogenous L-arginine. L-Arginine content (as measured via HPLC) was similar in young or older rats and the addition of exogenous L-arginine did not affect FMD in either young or older rats. Thus, old rats have ample microvascular L-arginine stores and the notion that insufficient substrate contributes to impaired NO-dependent vasodilatation with ageing is not a viable mechanism.

(c) Increased arginase activity. Arginase may act as a catalytic sink, converting L-arginine to L-ornithine and urea in the urea cycle instead of NO (Durante *et al.* 2007). Arginase also competes with NOS for L-arginine binding and sensitizes NOS to asymmetric dimethyl-L-arginine (ADMA), the endogenous inhibitor of NOS (Durante *et al.* 2007). Increased arginase levels/activity could therefore reduce NO-mediated vasodilatation by reducing substrate (i.e. L-arginine) and substrate binding.

FMD of harvested arterioles was determined following arginase inhibition with N^{Ω}-hydroxy-nor-L-arginine (NOHA). NOHA administration had no effect on age-associated differences in FMD. Arterioles from older rats still exhibited a 51% reduction in FMD. Thus, arginase does not appear to modulate microvascular FMD with ageing.

(d) Reduced cofactor supply. Tetrahydrobiopterin (BH_4) is a key cofactor required for NO synthesis. BH_4 binds to the heme group of the N-terminal oxidase domain of NOS, stabilizing the dimer molecule. Binding shifts NOS into a high spin state, increasing enzyme activity, and increasing the affinity of NOS for arginine. BH_4 may also accept electrons from a flavin in the C-terminus reductase domain of NOS during the synthesis of NO and L-citrulline, acting as an important redox agent. Without these actions, NOS becomes uncoupled from arginine oxidation, and superoxide is produced from the oxidase domain, leading to formation of peroxynitrite. Biosynthesis of BH_4 can occur by one of three pathways: (1) from GTP via the *de novo* synthetic pathway; (2) from sepiapterin via the salvage pathway; and (3) via recycling pathways (i.e. regeneration of BH_4 from BH_2 via dihydrofolate reductase) (Schmidt & Alp, 2007).

FMD of harvested arterioles was determined following the administration of sepiapterin, a precursor for BH_4 that has been shown to augment cofactor production provided exposure time is brief (Schmidt & Alp, 2007). The addition of sepiapterin increased FMD in arterioles of old rats while having no effect on arterioles of young rats. Although exogenous administration of sepiapterin significantly

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augmented FMD in aged vessels, dilatation did not attain values seen in young vessels. Arteriolar BH_4 content (also assessed with HPLC) was 60% lower in old rats compared to young rats. These data suggest that BH_4 plays a key role in reduced endothelial-dependent vasodilatation of the microvasculature with ageing.

Interpretations and conclusions

Reduced BH_4 has been implicated as a significant determinant of reduced NO bioavailability and concomitant conduit/resistance vessel dysfunction. Although this study was the first to eloquently note such an association in aged resistance vessels devoid of clinical and subclinical disease, several questions remain. This study would have been strengthened by documenting the efficacy of NOHA in inhibiting arginase activity. Arginase inhibition has been shown to augment microcirculatory vasodilatation in aged human vessels (Holowatz *et al.* 2006), and this is in disagreement with the present work of Delp *et al.* This begs the question, was arginase fully blocked in this study? Future research employing additional arginase inhibitors (i.e. 2(*S*)-amino-6-boronohexanoic acid, difluoromethylornothine (*S*)-(2-boron-ethyl)-L-cysteine) should replicate present findings.

Delp *et al.* chose to administer a BH_4 precursor (i.e. sepiapterin) and not BH_4, and the rationale for this selection remains unclear. This study would have benefitted by documenting an increase in BH_4 content of the aged vessels following sepiapterin administration. The salvage pathway is the primary mechanism for conversion of exogenous sepiapterin to BH_4, while BH_4 production in endothelial cells in response to shear stress is mediated by the *de novo* synthesis pathway (Schmidt & Alp, 2007). If the treatment normalized BH_4 levels to values seen in the young control vessels, yet FMD was still lower in old vessels as currently reported in the study, this would indicate that there was another mechanism contributing to impaired microvascular FMD with ageing. Indeed, previous studies have demonstrated that BH_4 supplementation restores human conduit vessel FMD in older individuals to values seen in younger individuals (Eskurza *et al.* 2005). If the treatment did not normalize BH_4 levels, this could suggest impairment in the salvage pathway. In summation, it is presently unclear whether the lack of ability of sepiapterin to completely restore FMD in older rats was due to an inability to fully restore BH_4 content, or whether other mechanisms in addition to reduced BH_4 contribute to the decline in FMD with age.

Delp and colleagues examined BH_4 content of whole vessels and not specifically endothelial cells. BH_4 is found in endothelial cells and vascular smooth muscle cells. Thus, the findings of Delp *et al.* cannot be strictly extrapolated to endothelial function. An interesting follow-up study will be to examine BH_4 content of aged endothelial-deneutered vessels *versus* endothelial cells in order to examine whether changes with age are specific to endothelial cells.

Oxidative stress with ageing may be the prime culprit in reducing BH_4 concentrations. Thus, interventions that reduce oxidative stress may have favourable effects on microvascular function with ageing. Recent studies note improvements in microvascular function in aged vessels following antioxidant supplementation with ascorbate (vitamin C) (Holowatz *et al.* 2006). However, the antioxidant capacity of ascorbate does not completely explain the efficacy of improved FMD as BH_4 can be oxidized by peroxynitrite to BH_3 and BH_2 at an extremely fast rate; 10-times faster than the reaction between ascorbate and peroxynitrite (Schmidt & Alp, 2007). Improved FMD following ascorbate supplementation may be due to its ability to stabilize BH_4 and prevent formation of BH_2 from the BH_3 radical by assisting in recycling to BH_4 (Holowatz *et al.* 2006). Other lifestyle modifications, such as regular aerobic exercise, may also hold promise in reducing oxiditative stress and augmenting BH_4 bioactivity, improving endothelial function with ageing (Eskurza *et al.* 2005).

The work by Delp *et al.* nicely demonstrated that arginase inhibition and L-arginine supplementation had no effect on microvascular FMD. Sepiapterin improved FMD of aged resistance vessels (probably by increasing BH_4), but did not completely abolish the ravages of old age. While an L-arginine bath was not the fountain of vascular youth, a sepiapterin bath was a splash in the right direction. Ultimately, the reduction of microvascular BH_4 content with ageing may be a key mediator of impaired FMD in resistance vessels.

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