**The cGMP degrading enzyme phosphodiesterase-5 (PDE5) in cerebral small arteries of older people**

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**Abstract**

Cerebral small vessel disease in deep penetrating arteries is a major cause of lacunar infarcts, white matter lesions and vascular cognitive impairment. Local cerebral blood flow (CBF) in these small vessels is controlled by endothelial-derived nitric oxide (NO) which exerts a primary vasodilator stimulus on vascular myocytes, via cytoplasmic cyclic GMP. Here we investigated whether the cGMP-degrading enzyme PDE5 is present in small penetrating arteries in the deep subcortical white matter of older people.

Frontal cortical tissue blocks were examined from donated brains of older people (N=42, 24M:18F, median age 81, range 59-100 y). PDE5, detected by immunohistochemical labelling, was graded as absent, sparse or abundant in vascular cells within small arteries in subcortical white matter (vessel outer diameter: 20-100 µm).

PDE5 labelling within arterial myocytes was detected in all cases. Degree of PDE5 expression (absent, sparse or abundant) was not associated with age or with neuropathological diagnosis of small vessel disease.

In conclusion, PDE5 is present in vascular myocytes within small penetrating arteries in older people. This is a potential molecular target for pharmacological interventions. [176 words]

**Keywords**

PDE5; cerebral blood flow; vascular cognitive impairment; arteriolosclerosis

**Introduction**

Cerebral small vessel disease (SVD) is associated with reduced cerebral blood flow (CBF) in deep brain regions. SVD is the most common cause of white matter lesions (WML), lacunar infarcts and cerebral microbleeds, and a significant contributor to vascular cognitive impairment (VCI) (1, 2). Nitric oxide (NO) produced by vascular endothelium plays crucial roles in regulating vascular tone and is a key regulator of CBF. Endothelial-derived NO has a vasorelaxant action in small arteries, stimulating guanylate cyclase activity in the cytoplasm of vascular myocytes. This leads to increased formation of the cytoplasmic messenger cyclic GMP (cGMP) and hence to vasodilation. The vasodilator effects of NO are curtailed by degradation of cytoplasmic cGMP by phosphodiesterase enzymes (such as PDE5) (3). In some vascular beds, e.g. the corpus cavernosum of the erectile tissue, PDE5 is the main cGMP degrading enzyme (3).

Reduced CBF is a well-established finding in SVD and in VCI (4). Increasing blood flow in the vasculature of the deep white matter and deep grey nuclei is an attractive hypothesis for slowing progression of VCI pathology (5). Pharmacological PDE5 inhibitors (such as sildenafil, Viagra®) are now widely used clinically to treat erectile dysfunction (ED) or pulmonary hypertension. PDE5 blockade, prolonging the action of NO, would be a plausible strategy to increase local CBF in deep brain areas affected by SVD(6). In the present study, we aimed to identify whether PDE5 protein expression is present in small penetrating arteries in the brains of elderly humans and whether PDE5 related to the presence of SVD.

**Methods**

We examined subcortical white matter tissue in a cohort of older people with documented neuropathological diagnosis of moderate-severe SVD (n=20) and aged control cases (n=22). See Table 1 for further details. Moderate-severe SVD and aged control cases were defined according to neuropathological criteria documented for all cases (1). Briefly, H&E sections were assessed by a registered neuropathologist. SVD was defined by vasculopathy-oriented criteria, including: hyaline thickening of arteriolar walls; widened perivascular spaces; parenchymal changes considered to result from SVD (perivascular pallor of myelin staining, loosening with attenuation of nerve fibres with gliosis in white matter or loss of nerve cells and gliosis in deep grey matter) in one or more sections (1, 7), see Supplementary Methods.

All cases had minimal AD neuropathology (Braak stage II or less) (7, 8). All donors were in the Oxford Project to Investigate Memory and Ageing (OPTIMA) cohort ([www.medsci.ox.ac.uk/optima](http://www.medsci.ox.ac.uk/optima)). Tissue was harvested with post-mortem delay <100 hours. This study had approval of the UK National Research Ethics Service. All tissue was donated following written informed consent by donors or next of kin.

Immunohistochemical labelling was performed as in our previous published work (7, 8) (see Supplementary Methods). Methods for defining neuropathological grades of SVD severity, vessel sclerotic index, degree of PDE5 labelling and radiological grade for white matter lesion load (derived from in-life CT scans)(9) are given in Supplementary Methods.

Contingency tables were compared using chi-squared tests. Differences between two experimental groups were tested using Mann-Whitney U-tests and between three or more groups using Kruskal-Wallis tests. P<0.05 was considered significant.

**Results**

Immunolabelling for PDE5 was detected in vascular myocytes of small arteries within deep subcortical white matter of older people (example in Fig 1). Some degree of myocyte PDE5 labelling was seen in all cases (N=42). Labelling was absent in neighbouring sections treated identically with an irrelevant primary antibody (rabbit anti-sheep IgG; Fig 1B). Neighbouring sections labelled with another PDE5 antibody (raised against a different immunogen) showed a qualitatively similar labelling pattern, with widespread labelling of vascular myocytes (Fig 1C). Vascular myocyte labelling was also seen in subcortical small arteries of young adults (age 18-40, n=3; example shown in Fig 1D).

As expected, immunolabelling for PDE5 was also seen in myocytes of small vessels within human corpus cavernosum and in Purkinje neurones within sections of cerebellar cortex (supplementary Figure S1). PDE5 labelling of vascular myocytic cells in brain vessels and in corpus cavernosum, as well as labelling of Purkinje neurones, were lost following immunodepletion of the primary antibody using the immunogenic peptide (supplementary Fig S1).

Older subjects with neuropathologically-defined SVD (1) were compared with aged control subjects (Table 1). The SVD group had lower cognitive scores (MMSE, CAMCOG) with higher incidence of clinical dementia than the control group (Table 1). Neuropathological SVD severity score(10), radiological white matter lesion score(9), arterial sclerotic index(7) and incidence of cerebral atherosclerosis were all greater in the SVD group than in aged controls (Table 1). The degree of brain vascular myocyte PDE5 immunolabelling was quantified using simple categorical grading (0=absent, 1=sparse, 2=abundant) for all small arteries within the size range 20-100µm. Among 42 cases examined, 5-10 vessels were scored for each case (median: 6 vessels per case, IQR 5.5, 7).The median PDE5 grade was “0” (absent) in 7 cases, “1” (sparse) in 26 cases and “2” (abundant) in 9 cases. Within this cohort, median PDE5 grade did not significantly differ between SVD cases and aged controls (see supplementary Fig S2). Median PDE5 grade was not significantly associated with age at death, with neuropathological markers of SVD severity (average sclerotic index, neuropathological rating of SVD severity) or with severity of radiological white matter lesions (Fig S2). Regarding sclerotic index, we also performed sensitivity analyses at the individual vessel level and found no trend (not shown).

**Discussion**

We explored PDE5 expression in vascular myocytes of small penetrating arteries within subcortical white matter of older people. We examined a well-characterised tissue cohort with detailed clinical and neuropathology data, selected for minimal Alzheimer’s disease pathology (7, 8). We aimed to identify whether PDE5 protein expression was present in the brain vessels of this population of elderly humans and whether its presence correlated with the level of vascular disease. PDE5 immunoreactivity was present in vascular myocytes in all cases examined. The degree of positivity was not related to age or to neuropathological indicators of SVD severity (average sclerotic index for small arterial vessels, or neuropathological rating of SVD severity) (7, 8).

Our findings accord with previous reports, demonstrating PDE5 mRNA and protein in brain tissue of humans and experimental animals (11-14). Western blot techniques detected PDE5 in lysates derived from meningeal and larger cerebral arteries of rodents and human patients (15-17).

Our study has several limitations. First, as our group sizes are small, the statistical power of the study is limited. It may be that assessment of a larger cohort would detect modest differences between SVD and aged control groups (see supplementary Fig S2). Second, we have deliberately excluded cases with Alzheimer’s disease (AD) pathology. Thus, we will miss possible interactions of SVD with AD vascular pathology. Third, we have not performed a comprehensive assessment of age-dependent PDE5 expression across all age ranges.

Potent selective PDE5 inhibitors (such as sildenafil, Viagra®) are now widely used clinically to treat ED and pulmonary hypertension. PDE5 blockade is a possible strategy to increase local CBF in deep brain areas affected by SVD(6). Clinical trials to examine effects of PDE5 inhibitors on brain perfusion are in progress within different patient groups (5, 18, 19). The current study demonstrates that the molecular target PDE5 is present in cerebral vascular myocytes, within the small arteries affected by SVD.

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**References**

1. Esiri MM, Wilcock GK, Morris JH. Neuropathological assessment of the lesions of significance in vascular dementia. J Neurol Neurosurg Psychiatry 1997;63:749-53

2. Ighodaro ET, Abner EL, Fardo DW, et al. Risk factors and global cognitive status related to brain arteriolosclerosis in elderly individuals. J Cereb Blood Flow Metab 2017;37:201-16

3. Conti M, Beavo J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem 2007;76:481-511

4. O'Sullivan M, Lythgoe DJ, Pereira AC, et al. Patterns of cerebral blood flow reduction in patients with ischemic leukoaraiosis. Neurology 2002;59:321-6

5. Pauls MMH, Clarke N, Trippier S, et al. Perfusion by Arterial Spin labelling following Single dose Tadalafil In Small vessel disease (PASTIS): study protocol for a randomised controlled trial. Trials 2017;18:229

6. Pauls MM, Moynihan B, Barrick TR, et al. The effect of phosphodiesterase-5 inhibitors on cerebral blood flow in humans: A systematic review. J Cereb Blood Flow Metab 2018;38:189-203

7. Giwa MO, Williams J, Elderfield K, et al. Neuropathologic evidence of endothelial changes in cerebral small vessel disease. Neurology 2012;78:167-74

8. Smallwood A, Oulhaj A, Joachim C, et al. Cerebral subcortical small vessel disease and its relation to cognition in elderly subjects: a pathological study in the Oxford Project to Investigate Memory and Ageing (OPTIMA) cohort. Neuropathol Appl Neurobiol 2012;38:337-43

9. Bridges LR, Andoh J, Lawrence AJ, et al. Blood-brain barrier dysfunction and cerebral small vessel disease (arteriolosclerosis) in brains of older people. J Neuropathol Exp Neurol 2014;73:1026-33

10. Mendes Ribeiro HK, Barnetson LP, Hogervorst E, et al. A new visual rating scale for white matter low attenuation on CT. Eur Neurol 2001;45:140-4

11. Menniti FS, Ren J, Coskran TM, et al. Phosphodiesterase 5A inhibitors improve functional recovery after stroke in rats: optimized dosing regimen with implications for mechanism. J Pharmacol Exp Ther 2009;331:842-50

12. Lakics V, Karran EH, Boess FG. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology 2010;59:367-74

13. Shimizu-Albergine M, Rybalkin SD, Rybalkina IG, et al. Individual cerebellar Purkinje cells express different cGMP phosphodiesterases (PDEs): in vivo phosphorylation of cGMP-specific PDE (PDE5) as an indicator of cGMP-dependent protein kinase (PKG) activation. J Neurosci 2003;23:6452-9

14. Teich AF, Sakurai M, Patel M, et al. PDE5 Exists in Human Neurons and is a Viable Therapeutic Target for Neurologic Disease. J Alzheimers Dis 2016;52:295-302

15. Kruuse C, Thomsen LL, Jacobsen TB, et al. The phosphodiesterase 5 inhibitor sildenafil has no effect on cerebral blood flow or blood velocity, but nevertheless induces headache in healthy subjects. J Cereb Blood Flow Metab 2002;22:1124-31

16. Kruuse C, Khurana TS, Rybalkin SD, et al. Phosphodiesterase 5 and effects of sildenafil on cerebral arteries of man and guinea pig. Eur J Pharmacol 2005;521:105-14

17. Kruuse C, Gupta S, Nilsson E, et al. Differential vasoactive effects of sildenafil and tadalafil on cerebral arteries. Eur J Pharmacol 2012;674:345-51

18. Lindberg U, Witting N, Jorgensen SL, et al. Effects of Sildenafil on Cerebrovascular Reactivity in Patients with Becker Muscular Dystrophy. Neurotherapeutics 2017;14:182-90

19. Lorberboym M, Makhline E, Lampl Y. Regional cerebral blood flow following single-dose and continuous-dose tadalafil after stroke. Acta Neurol Scand 2014;130:380-6

**Figure Legend**

Figure 1. PDE5 in small vessels of human subcortical brain tissue.

A, in an older person (male, age 82 y) vascular myocytes in a small penetrating artery within deep subcortical white matter are positive for PDE5 (brown, DAB chromogen. Arrow shows an example). B, these cells are negative in a neighbouring section treated with irrelevant primary antibody (rabbit anti-sheep IgG). C, a neighbouring section treated with a different PDE5 antibody, raised against a different immunogen, shows a similar labelling pattern. D, in a young adult (male, age 20 y) a small vessel within deep subcortical white matter is positive for PDE5. Sections in panel A and D were treated with a rabbit polyclonal antibody raised against a recombinant peptide from the C-terminal of human PDE5 (Cell Signalling Technology, Danvers, MA). Panel C was treated with a rabbit polyclonal antibody raised against a synthetic phospho-peptide corresponding to the N-terminal of human PDE5 (kindly supplied by Dr J Beavo and Dr S Rybalkin, University of Seattle, WA). Haematoxylin was used as a nuclear chromatin counterstain (Blue). Scale bars 20 µm.