*Highlights (for review)

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Method: We performed a systematic search of electronic databases (Medline and Embase). Our search terms were cerebral ischaemia, cerebral endothelial cells, cyclic nucleotide, phosphodiesterase and phosphodiesterase inhibitors.

Results: We found 23 publications which described effects of selective inhibitors of only three PDE families on endothelial function in ischemic stroke. PDE3 inhibitors (PDE3i) (11 publications) and PDE4 inhibitors (PDE4i) (3 publications) showed anti-inflammatory, anti-apoptotic or pro-angiogenic effects. PDE3i also reduced leucocyte infiltration and MMP-9 expression. Both PDE3i and PDE4i increased expression of tight junction proteins and protected the blood-brain barrier. PDE5 inhibitors (PDE5i) (6 publications) reduced inflammation and apoptosis. In preclinical models, PDE5i enhanced cGMP/NO signalling associated with microvascular angiogenesis, increased cerebral blood flow and improved functional recovery. Non-specific PDEi (3 publications) had mainly anti-inflammatory effects.

Conclusion: This review demonstrates that non-selective and selective PDEi of PDE3, PDE4 and PDE5 modulated endothelial function in cerebral ischemic stroke by regulating processes involved in vascular repair and neuroprotection and thus reduced cell death and inflammation. Of note, they promoted angiogenesis, microcirculation and improved functional recovery; all are important in stroke prevention and recovery, and effects should be further evaluated in humans.

Title: Cyclic nucleotide phosphodiesterases (PDEs) and endothelial function in ischaemic stroke. A review.

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Background

Endothelial dysfunction is a common feature in small vessel and large artery strokes. Stroke is the third leading cause of death and a major cause of disability (Sacco et al. 2013) in the Western countries. Early initiation of prophylactic treatment is warranted to reduce the impact as well as the risk of recurrence of this often-devastating disease. Though some signalling pathways in endothelial function are well-known, such as the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway, the underlying mechanisms for endothelial dysfunction precipitating a stroke may vary and they have yet to be fully resolved.

Diabetes, hypercholesterolaemia and hypertension are risk factors which cause both endothelial dysfunction and stroke. In these conditions the cerebrovascular response appears to be decreased and the vessels are more vulnerable to platelet adherence, blood-brain barrier breach, inflammation, and vasospasms (lRouhl et al. 2012; Wiseman et al. 2014; Ohtake et al. 2004). Modulation of endothelial function is a potential new treatment target, in both primary and secondary prevention of stroke (Pauls et al. 2018; Olmestig et al. 2017; Wardlaw 2010; Wardlaw et al. 2009). Current stroke treatments aim to dissolve blood clots and reduce platelet aggregation. However, targeting endothelial function may increase the collateral blood flow (Pantoni 2010; Pantoni, Fierini, and Poggesi 2014; Sandercock et al. 2012). Also, the blood-brain barrier (BBB) homeostasis, important in reducing an inflammatory or toxic response in the brain tissue, requires an intact endothelial function. With diabetes, hypertension and hypercholesterolemia the endothelium becomes non-responsive to vascular cues, increases the pro-inflammatory response, and may turn pro-thrombotic (Yau, Teoh, and Verma 2015). The underlying cellular processes which render the endothelium less responsive to stimuli and which reduce endothelial cell integrity need to be explored to identify new treatment targets. These processes are known to involve the cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cGMP, intracellular second messenger molecules (Kolluru et al. 2008; Ogawa et al. 1992; Draijer et al. 1995; Stelzner Thomas, Weil, and O'Brien 1989). Cyclic nucleotide signalling contributes to regulation of the expression of endothelial adhesion molecules, activation of smooth muscle cell function, and is involved in receptor mediated signalling (Fukuhara et al. 2005; Waschke 2008; Yuan and Rigor 2010) (Figure 1). Cyclic nucleotide levels are regulated through their production by cyclases, by efflux, and through degradation by cyclic nucleotide phosphodiesterases (PDE) (Keravis and Lugnier 2012; Conti and Beavo 2007; Beavo 1995; Sassi et al. 2012). Changing the cyclic nucleotide signalling in the endothelial cell affects vasoreactivity (Figure 1).

Excessive production of reactive oxygen species and low-grade inflammation can precede disease progression and further add to the dysfunction of the cerebral endothelium. Increased chemokine and cytokine secretion, caused by vascular injury, attracts the passage of leucocytes from the blood into the brain and contributes to the development of cellular and vasogenic oedema with sustained inflammation and a disturbed cerebral microcirculation (Takeshita et al. 2014; Pober and Sessa 2015). In a stroke, several mechanisms of action may thus be targeted: platelet aggregation, flow changes induced by cardiac arrhythmia or stenosis of the arteries, or changes in the vessel walls (Moskowitz, Lo, and Iadecola 2010). PDEs are potential therapeutic targets in all these mechanisms. We need to fully uncover the role of PDEs in the cells which are cardinal to the blood-brain barrier function and to the expression of adhesion molecules associated with the prevention and treatment of ischemic stroke and related tissue damage. Several PDE inhibitors (PDEi) are available for clinical use in humans, but so far only two, cilostazol and dipyridamole, are approved for the secondary prevention of stroke and both are considered to mainly inhibit platelet aggregation (Igawa et al. 1990).

In this review, we investigate current literature on the role of PDEs and related cyclic nucleotides in cerebral endothelial function and dysfunction. Further, we disclose the underlying mechanisms of how PDEi may amend endothelial signalling pathways involved in protecting the integrity and function of cerebral endothelium during or after ischemic stroke. We wish to highlight which PDEi may be relevant for both treatment and prevention of endothelial dysfunction and stroke.

Methods

We followed Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (Moher et al. 2015). Employing a systematic literature search of Medline and Embase, we identified relevant publications with last search done 31st January 2019. Search terms were cerebral ischemic stroke OR cerebral endothelium AND cyclic nucleotide phosphodiesterase OR phosphodiesterase inhibitor. The selection was based on pre-determined criteria. Two investigators independently screened the titles and abstracts for eligibility (S.Y. and B.H.A). In cases of disagreement, consensus was achieved by a third investigator (C.K.). A subsequent screening was done by reading the full text to comply with the inclusion and exclusion criteria (S.Y). **Inclusion criteria** were studies that investigated the role of PDEi in the cerebral endothelial function in each of the following: ischemic stroke patients, animal ischemic stroke models or in-vitro endothelial cell models. **Exclusion criteria** were studies that investigated the role of PDEi in tissues and cells

outside the brain, the role of PDEi in cerebral cells other than endothelial cells, which included non-ischemic stroke types, non-English papers, and reviews.

In addition to the systematic search for PDEi effects on cerebral endothelium, the currently available data on the expression of PDEs in the cerebral microvessels in general was included.

Results

With the systematic database search, we retrieved 677 non-duplicate papers. After reviewing titles and abstracts we identified 38 papers eligible for full text screening according to the inclusion and exclusion criteria. Subsequently, 15 papers were excluded for the following reasons: the papers reported the effect of PDEi or cyclic nucleotides in non-cerebral endothelial cells or in non-vascular brain cells or did not apply PDEi. As a result, 23 papers in total met the inclusion criteria for this review. A flow chart for selection of papers is shown in the PRISMA flow chart (Figure 2).

Overview of the distribution of PDEs in the cerebral vasculature

So far 11 PDE families have been identified, each with unique cAMP and/or cGMP degrading functions, variable mechanisms of regulation, as well as cell and tissue specific distribution (Keravis and Lugnier 2012). The expression data in Table 1 summarize which PDEs have been characterized in tissue and isolated cells from human, rat and mouse brain, in addition to the findings in mouse and human cerebral endothelial cell lines, bEND.3 and hCMEC/D3. In contrast to PDEs in brain tissue (Kleppisch 2009), the distribution of PDE families throughout the cerebral vascular tree is little studied. There is a considerable knowledge gap in how each of the cell types in the neurovascular unit, endothelial cells, smooth muscle cells, astrocytes, and pericytes, contributes to the regulation of endothelial function and brain microperfusion. Most studies report on brain homogenates rather than isolated cell types. Further, the cerebral endothelial cell layer is fragile, and they rapidly deteriorate post mortem or during the preparation for experimental procedures rendering it difficult to identify the function of this specific cell type (Onoue et al. 1993).

PDE activities were initially measured in cerebral microvessels isolated from bovine cortex (Stefanovich 1979), where both cGMP and cAMP hydrolytic activity was detected.

All PDE families are expressed in the human brain, but differential expression of the PDE families and their isoforms in specific brain areas or cell types contribute to their versatile functions in cell signalling (Lakics, Karran, and Boess 2010). There is little information on the expression and function of the PDE families specifically in brain microvascular endothelial cells.

PDE1. The PDE1 family was one of the first to be purified from bovine brain homogenate, identifying a PDE enzyme which was activated by Ca^{2+}/calmodulin and with a high affinity for cAMP (Morrill, Thompson, and Stellwagen 1979). PDE1 is now known to be abundantly expressed in the mammalian brain (Sharma and Kalra 1994) and includes the three isozymes PDE1A, PDE1B and PDE1C. Expression pattern varies between brain regions and even within the same type of Purkinje cells (Bender and Beavo 2006; Beavo 2007). In large cerebral arteries of both guinea pig and man, PDE1A and PDE1B protein and corresponding PDE1 hydrolytic activity was detected (Kruuse et al. 2005; Kruuse et al. 2001).

PDE2. Expression of PDE2A was detected in the bovine brain (Sonnenburg and Beavo 1994). In human brain tissue, PDE2A mRNA was present in the hippocampus and all cortical regions (Lakics, Karran, and Boess 2010). PDE2A protein was expressed in endothelial cells of capillaries supplying the human cerebellum, and in cell homogenates showed that protein expression was highest in the cerebellar cortex while it was lower in the cerebellum (Sadhu et al. 1999). The most abundant expression of PDE2A mRNA was in the human forebrain (Stephenson et al. 2009b).

In the human cerebral microvascular endothelial cell line (hCMEC/D3), a widely used *in vitro* cell model for BBB experiments, most PDE isoforms (excluding PDE1, PDE2B, PDE3B, PDE9B, PDE11) were present at the mRNA level (Table 1) (Kalari et al. 2016).

PDE3 and PDE4. Little is known of the specific cellular distribution of PDE3 and PDE4 in cerebral arteries and microvessels (Kawanabe et al. 2012; Kelly et al. 2014). The hydrolytic activity of both PDE3 and PDE4 was detected in homogenized guinea pig and human large cerebral arteries; only PDE4 effects appeared endothelial dependent (Birk et al. 2004). These findings confirmed the results which showed hydrolytic activity of PDE4 in canine cerebral arteries (Willette et al. 1997).

PDE5. In the human brain, two PDE5 isoforms, PDE5A1 and PDE5A2, were expressed at the mRNA and protein level. PDE5A1 and PDE5A2 mRNA expression was ubiquitous, PDE5A3 expression was found to be specific to the smooth muscle cells (Lin 2004). In human brain tissue sections, expression of PDE5 protein has been detected in cerebral smooth muscle cells (Vasita et al. 2019). PDE5A protein and hydrolytic activity was reported in homogenates of guinea pig and human large cerebral arteries (Kruuse et al. 2005; Kruuse et al. 2001).

The effect of the PDE deficiency on cerebrovascular outcome or endothelial function was not investigated in the various studies of PDE knockout animals and cell lines (Assenza et al. 2018; Brennenstuhl et al. 2015; Choi et al. 2006; Li et al. 2011; Pathak et al. 2016; Siuciak et al. 2006; Yang et al. 2003; Cygnar and Zhao 2009; Ehrman et al. 2006; Lee et al. 2015; Sun et al. 2007; Zhang

et al. 2008), but it is possible that some PDE knockout animals may not be viable due to vascular dysfunction, though such needs to be confirmed.

PDEi in endothelial function during cerebral ischaemia

Though specific inhibitors for some PDE families such as PDE3i, PDE4i, and PDE5i are currently commercially available and applicable in humans, so far there is a lack of specific inhibitors for the remaining PDE families. Further, the effect of PDEi on cerebral endothelial function are only reported for the inhibitors of these three PDE enzymes.

A key approach to better treatment strategy of stroke injury needs to be combined therapy which inhibits platelet aggregation and enhances blood perfusion in ischemic stroke regions (Moskowitz, Lo, and Iadecola 2010). Briefly, as a reaction to a stroke injury the brain responds with activation of the immune system, and neuroinflammation is initiated. Damage of the cerebral vascular endothelium, which is a basic element of the BBB, triggers expression of adhesion molecules and mediates infiltration of immune cells into the brain parenchyma (Lakhan, Kirchgessner, and Hofer 2009). Platelet aggregation and consequent occluded vessels result in insufficient supply of oxygen and nutrients to brain regions, leading to cerebral ischaemia characterized by hypoperfusion and loss of neuronal tissue. Therefore, an optimal therapeutic approach for stroke needs to be tailored to cover anti-inflammatory, anti-apoptotic and pro-angiogenic effects for neuro-reparative and neuroprotective effects.

PDE3 inhibitors

In total, eleven studies investigated the effect of PDE3 inhibition on cerebral endothelial function using cilostazol (listed in Table 2). Two of the studies (Takeshita et al. 2014; Horai et al. 2013) investigated effects of cilostazol by measuring the transendothelial electrical resistance (TEER) in a triple co-culture cell model. The triple co-culture cell model is an *in vitro* blood-brain barrier model that mimics the neurovascular unit which comprises endothelial cells, astrocytes and pericytes. TEER is a surrogate marker for the integrity of the BBB and is used to determine the endothelial tightness in response to treatment. TEER values were, compared to placebo, significantly higher when cilostazol was applied in a co-culture cell model using primary rat brain endothelial cells, astrocytes, and pericytes, exposed to oxygen glucose deprivation (OGD)/3-hour reoxygenation, and

treated with cilostazol (1 µM) (Horai et al. 2013). This suggested a preservation of the brain barrier function and integrity of the rat brain endothelial cells after cilostazol treatment during conditions mimicking a stroke (Horai et al. 2013). Accordingly, cilostazol (10 µM) treatment resulted in a significant increase in TEER in a similar co-culture cell model exposed to OGD/24-hour reoxygenation, compared to normoxia/reoxygenation control cells in the presence of advanced glycation end products (AGE) indicative of a barrier dysfunction (Takeshita et al. 2014). AGEs form when protein or fat is glycosylated after exposure to high glucose in the blood, and they accumulate in the endothelial cell walls, trigger low-grade inflammation and contribute to diabetic vascular complications such as stroke (Wautier and Schmidt 2004). In addition, expression of claudin-5 (cld-5), a tight junction protein connecting endothelial cells, was restored in response to cilostazol treatment compared with vehicle treatment cells exposed to OGD/AGE (Takeshita et al. 2014). Thus, PDE3 inhibition helps maintain the integrity of the endothelial barrier function during ischemic conditions in cell models.

Further to this, the effects of cilostazol on the integrity of endothelial cells in the neurovascular unit were investigated in stroke-prone spontaneously hypertensive rats (SHR-SP) (Omote et al. 2014). Animals were allocated to four different groups and treated for 14 days with vehicle, cilostazol, the more widely used antiplatelet agents in stroke (Rothwell et al. 2016; Diener, Ringleb, and Savi 2005) acetylsalicylic acid, or clopidogrel. Cilostazol treatment reduced the expression of matrix metalloproteinase-9 (MMP-9), an enzyme which degrades extracellular matrix, and inhibited the detachment of astrocytes and pericytes from endothelial cells, reducing the infarct volume (Omote et al. 2014). Matrix metalloproteinases disrupt tight junctions and increase BBB permeability post stroke (Cunningham, Wetzel, and Rosenberg 2005). Histological analysis of brain tissue from rats treated with cilostazol showed an increased expression of vascular endothelial growth factor receptor 2 (VEGFR-2) compared to tissue derived from rats receiving acetylsalicylic acid, clopidogrel, or vehicle. Increased VEGF expression is correlated with local brain angiogenesis and a corresponding up-regulation of VEGFR-2 on cerebral endothelial cells post stroke (Zhang et al. 2002).

Patients with diabetes and cerebral ischaemia who were treated with cilostazol responded with an increased level of circulating endothelial progenitor cells (EPC), (Ueno et al. 2011) which are important for maintenance and repair of injured vessels (Rafii and Lyden 2003).

Haemorrhagic transformation in the infarcted area is a potentially devastating side effect of thrombolytic therapy. Thrombolysis, performed by infusion of tissue plasminogen activator (tPA) which dissolves the embolus, can induce hemorrhagic transformation due to disintegration of cerebral microvessels over time. It is required that tPA is administered as early as possible,

preferably within 4.5 hours from stroke onset to reduce the risk of hemorrhagic transformation and intracerebral bleeding. If cilostazol protects endothelial integrity, the time window for thrombolysis could be expanded by concomitant administration of cilostazol and tPA, and more patients may be treated (Gravanis and Tsirka 2008). Mice subjected to both transient middle cerebral artery occlusion (tMCAO) and reperfusion were fed with food containing cilostazol, acetylsalicylic acid or normal diet for 7 days prior to induction of ischaemia. Hemorrhage lesions in the ischemic area were assessed 24 hours after induction of ischaemia. tPA was administered immediately before the reperfusion (Kasahara et al. 2012). Cilostazol significantly reduced the tPA-induced cerebral hemorrhagic transformation in the ischemic area after 90 min and 120 min of ischaemia (Kasahara et al. 2012). Further, in cilostazol-treated mice the platelet endothelial cell adhesion molecule 1 (PECAM), which is essential for the function, structure and survival of endothelial cells (Park et al. 2010), was not reduced. Also, pretreatment with cilostazol, compared to acetylsalicylic acid, protected the tight junctions and basement membranes in the ischemic area. Thus, cilostazol prevented degradation of the cerebral microvessels and protected the structural integrity through reduction of MMP-9 after cerebral ischaemia (Kasahara et al. 2012).

In a murine model of transient focal cerebral ischaemia of 45 or 90 minutes, mice were treated with cilostazol or vehicle for 7 days. tPA or placebo was administered prior to a 24-hour reperfusion (Hase et al. 2012). Treatment with cilostazol without tPA improved blood flow and reduced expression of endothelial adhesion molecules involved in leukocyte infiltration and platelet adhesion after acute ischemia. Further, brain oedema and hemorrhagic transformation was significantly reduced after 45- and 90-min ischaemia with a decreased density of MMP-9 positive microglia. Cilostazol, both with and without tPA treatment significantly improved neurological outcome, suppressed the local re-flow, and improved microvascular integrity (Hase et al. 2012).

Inducing tMCAO for two, three, and six hours in a similar murine model, followed by tPA and cilostazol or vehicle infusion prior to reperfusion, the combined cilostazol/tPA treatment (Ishiguro et al. 2011) ameliorated the hemorrhagic transformation at six hours. Cilostazol further reduced brain oedema, decreased MMP-9 activity and prevented a reduction in claudin-5 expression compared to tPA therapy alone (Ishiguro et al. 2011).

Application of tPA to a co-culture of endothelial cells, pericytes and astrocytes induced NVU damage, which was reversed by a high dose of cilostazol (100 µM). Cilostazol also significantly reduced cellular injury of the pericytes but not the astrocytes. Addition of a cAMP analogue at concentrations of 300 and 1000 µM to the cell co-culture showed that the tPA-induced vascular damage was mitigated (Ishiguro et al. 2011).

In patients with atrial fibrillation, a major risk factor for stroke, the use of anti-coagulation therapy (warfarin or new oral anticoagulation, NOAC) for stroke prevention is associated with a higher risk of intracerebral hemorrhage or hemorrhagic transformation of ischemic brain tissue. Cilostazol, administered immediately after reperfusion, alleviated the warfarin-induced hemorrhagic transformation in mice treated with warfarin prior to three hours of tMCAO followed by 21 hours of reperfusion (Kitashoji et al. 2013). Cilostazol reduced bleeding time and increased the survival rate in the group receiving the dual therapy. Further to this, cilostazol increased the expression of tight junction proteins: zonula occludin (ZO-1), claudin-5, and the vascular endothelial cadherin (VE-cadherin). This suggested that cilostazol protected the vascular endothelial integrity (Kitashoji et al. 2013).

A key feature in endothelial dysfunction related to ischaemia is the adherence of platelets and leucocytes to endothelial cells in cerebral microvessels. One group investigated whether cilostazol inhibited the rolling and adhering of platelets in cerebral microvascular endothelial cells after transient ischemia. Mice were subjected to transient cerebral ischaemia by occlusion of bilateral common carotid arteries (BCCA) for 15 min followed by reperfusion. Cilostazol was administered 30 min prior to the BCCA occlusion and compared to a control group with no ischaemia and no reperfusion (Fukuoka et al. 2014). At three and six hours post ischemia, the group of mice pretreated with cilostazol showed a reduced number of rolling and adhering of platelets to endothelial cells, compared to what was observed in the ischaemia reperfusion group and the control group (Fukuoka et al. 2014). This showed that cilostazol inhibited platelet adherence to vessel walls, thus reducing an inflammatory response after ischemia. Hemorrhagic transformation was not observed in the ischemic area when treated with cilostazol.

Patients with non-cardioembolic ischemic stroke, enrolled within a week from stroke onset, were evaluated to determine if acetylsalicylic acid with or without cilostazol co-medication reduced platelet aggregation and endothelial markers in the blood. Platelet aggregation, induced by collagen, adenosine diphosphate or arachidonic acid, decreased significantly in pre-treatment samples, with no change between the groups receiving acetylsalicylic acid alone or in combination with cilostazol. There were no investigations into the effect on endothelial function (Ohnuki et al. 2017).

In SHR-SP rats fed on chow with acetylsalicylic acid, cilostazol, or vehicle for five weeks prior to induction of transient ischemia, the cilostazol-treated rats showed a higher regional cerebral blood flow (rCBF), and a reduced infarct size compared to rats fed on acetylsalicylic acid or vehicle (Oyama et al. 2011).

PDE4 inhibitors

Three studies explored the role of PDE4 inhibition on endothelial function in cerebral microvessels and PDE4 inhibitor effects during cerebral ischemia. CD34, a transmembrane phosphoglycoprotein, is a marker for the EPC involved in angiogenesis (Kao et al. 2008). The change in EPCs was investigated in rats subjected to tMCAO for two hours followed by reperfusion and treatment with the PDE4 inhibitor, rolipram, from the first day of ischaemia and continued for three, seven or 14 consecutive days. Analysis of tissue sections from the ischemic brains showed that rolipram attenuated apoptosis of neuronal cells in the peri-infarct region, and enhanced the cell proliferation of microvessels by recruiting CD34-positive cells to the ischemic tissue, thereby contributing to angiogenesis (Hu et al. 2016). Furthermore, the infarct size was reduced, and the functional outcome was improved in rats treated with rolipram compared to vehicle-treated rats.

The involvement of PDE4 in the BBB and the increased vascular permeability induced by ischemic stroke has been a focus of attention. In rats subjected to transient ischaemia and treated with the PDE4 inhibitor BBB022 or vehicle (Belayev et al. 1998), the neurological scores and thus functional outcome was significantly improved by BBB022 compared to vehicle-treated animals (Belayev et al. 1998). Animals treated with BBB022 showed significantly reduced BBB vascular leakage compared to vehicle-treated animals. In addition, treatment of porcine and rat brain endothelial cell monolayers with PDE4 inhibitor rolipram or BBB022 increased the cAMP levels and improved cellular tightness. Thus, inhibition of PDE4 protected the blood-brain barrier integrity by lowering the vascular permeability after cerebral ischaemia (Belayev et al. 1998).

When injured, the endothelium releases pro-inflammatory mediators, which activate migration of immune cells into the cerebral vasculature augmenting the tissue damage (Anrather and Iadecola 2016). In mice with induced transient ischemia, rolipram caused higher expression of claudin-5 and occludin. In addition, they observed a reduced leukocyte infiltration through the endothelial cell layer and a decrease in the pro-inflammatory cytokines IL-1β and TNFα (Kraft et al. 2013). Further, a reduction was noted in infarct volume and oedema formation in rolipram-treated compared to non-treated mice.

PDE5 inhibitors

The impact of PDE5 inhibition on endothelial function was investigated in six studies: four studies (Zhang et al. 2003; Charriaut-Marlangue et al. 2014) (Li et al. 2007, Zhang R. 2005) applied

sildenafil; one study, a novel PDE5 inhibitor, DA-8159 (Choi, Kim, and Kang 2006); and one study used tadalafil (Zhang et al. 2006).

In rats subjected to one-sided common carotid artery occlusion and hypoxia for 120 min, followed by administration of either sildenafil or vehicle, brain sections were analyzed for integrity of microvessels, cell death and endothelial vessel density in addition to blood flow measurements (Charriaut-Marlangue et al. 2014). The glucose transporter 1 (Glut-1) is responsible for entry of glucose into endothelial cells and is used as a marker for changes in BBB capillary density. Sildenafil-treated rats had increased Glut-1 staining after ischaemia induction, indicative of preserved capillary density and branching. Sildenafil treatment was able to increase the mean blood flow velocity in rats with significant lesions after ischaemia and the effects were associated with improved motor function. In addition, sildenafil treatment resulted in reduction of astrocyte and microglial activation during ischemia. (Charriaut-Marlangue et al. 2014).

In a rat model of embolic MCA stroke, either sildenafil or a NO donor was administered 24 hours after stroke onset (Zhang et al. 2003). They demonstrated that rats treated with either sildenafil or NO donor showed significant increase in VEGF levels in tissue homogenates and induced capillary-like tube formation. They showed that NO enhanced angiogenesis in the ischemic brain via a NO/cGMP dependent pathway and that inhibition of PDE5 with sildenafil promoted the angiogenic process (Zhang et al. 2003). Supplementary to this, using the rat embolic stroke model with tadalafil administered 24 hours after stroke onset (Zhang et al. 2006), an increased proliferation of endothelial cells and vascular density was detected in the ischemic penumbra when compared to saline-treated rats. Tadalafil-treated rats also had significantly increased proliferation of subventricular zone cells, the neural progenitor cells, which suggested an increased neurogenesis. This effect may contribute to the improved functional recovery reported in this study after tadalafil treatment (Zhang et al. 2006).

The effects of a novel PDE5 inhibitor (DA-8159) were investigated on the endothelial function in SHR-SP rats. (Choi, Kim, and Kang 2006). The animals were allowed free access to a high-salt diet (containing 4% NaCl) to accelerate induction of hypertension, which is a risk factor for stroke. DA-8159 was administered daily and endothelial function was assessed: nitric oxide (NO), nitric oxide synthase (NOS), cGMP, P-selectin, endothelin-1, and the total anti-oxidative status. This study reported a significant increase in cGMP and NO levels, along with an improvement of the total anti-oxidative status and a decreased stroke lesion size in DA-8159-treated rats, compared to vehicle-treated rats (Choi, Kim, and Kang 2006).

Following induction of embolic stroke, aged and young rats were treated with sildenafil or vehicle (Zhang et al. 2005). Aged rats treated with sildenafil responded with a significantly improved functional recovery concomitant to higher cGMP levels, increased synaptogenesis and angiogenesis of endothelial cells compared to vehicle-treated controls. In general, vascular density and endothelial cell proliferation after stroke was attenuated in aged compared to young rats. In the embolic stroke model, sildenafil treatment resulted in long-term effects on stroke recovery, which were reported even after 6 weeks (Li et al. 2007). Sildenafil treatment of rats with embolic stroke partly enhanced the level of CBF, which, related to increased angiogenesis in the ischemic boundary area and, significantly improved functional recovery, compared with vehicle-treated animals (Li et al. 2007).

Other selective PDEi: Expression of the remaining PDE families other than PDE 3, 4 and 5 in cerebral vascular cells (Table 1) opens the possibility that they may also possess therapeutic potential to amend endothelial function. Though specific inhibitors are being developed, none are yet commercially available for human use and none have been reported to have effects on cerebral endothelial cells. Further studies are warranted to explore this interesting area of PDE effects.

Non-Specific PDEi

The non-selective and non-specific PDEi, dipyridamole (DP), used in secondary prevention of stroke based on its antithrombotic effects, was evaluated for effects on endothelial function in three studies (Guo et al. 2010; Zhao et al. 2006; Hallevi, Hazan-Halevy, and Paran 2007). The main outcome measured in the studies was the adhesion of neutrophils to an endothelial cell line and the expression of adhesion molecules on the endothelial surface, which contributes to leukocyte extravasation.

Neutrophils are a subclass of leucocytes which play an important role in the innate immune response (Rosales et al. 2016). Blood was retrieved from 12 stroke patients within 48 hours of stroke onset: from six patients with previous stroke, and from six healthy controls. Neutrophils were isolated from the blood and subsequently incubated with dipyridamole, the antihypertensive drug candesartan, an angiotensin II antagonist (Doggrell 2004), or a mix of dipyridamole and candesartan (Hallevi, Hazan-Halevy, and Paran 2007). Brain microvascular endothelial cells were incubated with the drug-treated neutrophils. The neutrophils from acute stroke patients treated with any of the drugs, alone or in combination, inhibited adhesion to the endothelial cells, and additional inhibition of neutrophil-adhesion to endothelial cells was observed with the dual treatment regime. Incubation of neutrophils from patients with chronic stroke or healthy controls, with dipyridamole or candesartan, did not reduce the adhesion of neutrophils to endothelial cells, though the authors reported a small non-significant effect of the combined treatment in both groups.

Furthermore, expression of the adhesion molecule macrophage antigen 1 (Mac-1) on neutrophils from patients with acute ischemic stroke after treatment with dipyridamole alone or combined with candesartan was reported to be significantly reduced, when compared to vehicle-treated neutrophils (Hallevi, Hazan-Halevy, and Paran 2007). This suggests that dipyridamole may have more than just anti-platelet effects in stroke prevention.

Effects of dipyridamole were evaluated in a human brain microvascular endothelial cell line exposed to 1) inflammatory stress by incubation with tumor necrosis factor alpha (TNF-α) after serum deprivation and 2) metabolic stress by OGD for 6 hours, followed by reperfusion for 18 hours (Guo et al. 2010). Dipyridamole treatment (1-5 μM) reduced the TNF-α induced expression of ICAM-1 and MMP-9. Also, dipyridamole significantly reduced cell death and MMP-9 levels after the metabolic insult. In the *in vitro* experiments, dipyridamole was reported to show both anti-apoptotic and anti-inflammatory effects in the endothelial cells (Guo et al. 2010).

The effect of dipyridamole, clopidogrel and acetylsalicylic acid on endothelial function was evaluated by measurements of NO, cGMP and von Wille brand factor (vWF) in plasma samples from ischemic stroke patients and healthy controls (Zhao et al. 2006). Also, C-reactive protein and platelet derived growth factor was measured in serum. A small cohort of patients (n = 11) and healthy controls (n = 11), with a history of ischemic stroke, were included within five years from the stroke incident. Both patients and controls received the drugs daily over a 2-week period, either as single medications, in pairs (acetylsalicylic acid/clopidogrel, acetylsalicylic acid/dipyridamole, or clopidogrel/dipyridamole) or altogether. No changes in cGMP or NO levels in plasma were reported using any drug or the subject groups. However, a significant decrease in vWF was observed in both controls and patients when treated with dipyridamole. vWF is a pro-coagulator glycoprotein released from injured endothelial cells, which promotes thrombus formation and platelet adhesion on the endothelial surface (Dhanesha et al. 2016) and is a marker of endothelial dysfunction. Recent studies suggest a role for vWF in regulating angiogenesis (Starke et al. 2011). The C-reactive protein level was reduced in patients treated with dipyridamole. Clopidogrel reduced plasminogen activator inhibitor-1 in patients (non-significantly) and in controls (significantly). The platelet-derived growth factor level was reduced in controls but not patients when treated with acetylsalicylic acid. Treatment with triple anti-platelet treatment did not result in any additive effect on the vascular parameters measured, compared with mono or dual treatment. Triple anti-platelet treatment reduced levels of vWF in both patients and controls, which was not reported with either clopidogrel or acetylsalicylic acid.

Another non-selective PDEi, theophylline, has recently been tested as an add-on agent to thrombolysis, as it is assumed to reduce the reperfusion damage and improve the final outcome in stroke patients (Modrau et al. 2016), and clinical results are underway.

Discussion

We explored, through a systematic search, studies investigating the effects of PDEi on endothelial function and dysfunction in ischemic stroke. The non-selective PDEi dipyridamole and the selective PDE3 inhibitor cilostazol are both currently used in secondary prevention of stroke based on their anti-platelet effects. However, the included studies reveal that other important mechanisms of action of PDEi, including effects on endothelial function, may apply to prevent strokes or reduce stroke outcome. PDEi are thus likely candidates to further target vascular changes related to ischemic stroke.

A crucial strategy for a better stroke treatment requires a multimodal approach which inhibits platelet aggregation and enhances blood perfusion in ischemic stroke regions (Moskowitz, Lo, and Iadecola 2010; Lin and Sanossian 2015). Briefly, as a response to the endothelial damage and cerebral ischaemia related to a cerebral vessel occlusion by an embolus or local thrombus, the brain tissue reacts by activating the immune system and neuroinflammation (Lakhan, Kirchgessner, and Hofer 2009). Injury to the cerebral vascular endothelium, affecting the BBB, triggers expression of adhesion molecules and mediates infiltration of immune cells into the brain parenchyma (Lakhan, Kirchgessner, and Hofer 2009). Thus, the optimal therapeutic approach in stroke treatment needs to be tailored to improve microcirculation and modulate anti-inflammatory, anti-apoptotic, and pro-angiogenic processes to achieve neuro-reparative and neuroprotective effects.

PDEi are promising tools to amend endothelial dysfunction and prevent cellular damage in ischemic stroke, though most data derive from *in vitro* experiments based on cells and animal studies, with inherent translational problems to humans.

The key PDE families currently shown to be involved in endothelial signalling in stroke are PDE3, PDE4 and PDE5, which are involved in promoting vessel formation, anti-apoptotic and anti-inflammatory pathways. The major PDEi tested in stroke models are the inhibitors of PDE3 (cilostazol), PDE4 (rolipram) and PDE5 (dipyridamole, sildenafil, tadalafil). In patients, only one PDEi with an effect on clinical outcome in stroke patients been tested. Administration of cilostazol combined with other conventional anti-thrombotics in acute stroke patients was associated with good

clinical outcome and recurrence three months after stroke onset (Fujimoto et al. 2016; Shinohara et al. 2010), but additional clinical studies are warranted.

PDE4 inhibitors have a narrow therapeutic window due to the associated to side effects such as headache, nausea and vomiting (Rutten et al. 2008). Mutations in the PDE4D gene have been associated with a higher susceptibility to ischemic stroke, in particular the cardioembolic stroke subtype, though results are ambiguous (Jørgensen et al. 2015; Song et al. 2006; Milton et al. 2011; Bevan et al. 2008; Wang et al. 2018; Kuhlenbäumer et al. 2006). A recent *in vivo* study in rats (Chen et al. 2018) used a modified PDE4 inhibitor with strong anti-inflammatory effects. The same drug showed no emetic side effects in beagle dogs, which warrants further studies to support a possible role of this modified PDE4 inhibitor in human stroke treatment. The PDE4D gene or associated pathways may still be an interesting therapeutic target in stroke, but the complexity of the physiological effects needs to be investigated.

The use of PDE3 inhibitors (e.g. cilostazol) to improve endothelial function and increase BBB tightness in animal models of stroke or rat/human endothelial cell models (triple co-culture to mimic neurovascular unit) show promise for more targeted treatment in humans with altered BBB function. In addition, cilostazol inhibited adhesion of platelets to endothelium post-ischaemia, mitigated the inflammatory reaction, and reduced expression of adhesion molecules and MMP-9. Of major clinical importance is that cilostazol seems to attenuate the tPA-induced hemorrhagic transformation, thus opening a possibility for increasing the time window for tPA treatment if combined with PDE3 inhibition.

The protective effect of cilostazol on hemorrhagic transformation may be through the expression of tight junction proteins that connect the ECs and maintain their integrity. AGE products are involved in diabetic vascular injury and contribute to ischaemia–reperfusion injury. As cilostazol was shown to alleviate the damage incurred by AGE, cilostazol may eligible as an add-on therapy in stroke patients with diabetes. Cilostazol also restored expression of tight junction proteins and ameliorated the damaging effects of AGE on integrity of the BBB, though these data need to be confirmed. The preclinical studies also suggested that the therapeutic time window of tPA could be extended by prior or concomitant administration of cilostazol.

The effect of PDEi on angiogenesis is interesting. Application of PDE4i in rats subjected to tMCAO showed enhanced angiogenesis reflected by differentiation of CD34+ cells to vascular endothelial cells, thus contributing to endothelial renewal/rejuvenation. Further, a reduction in neuronal apoptosis was observed in response to PDE4 inhibitor treatment (Hu et al. 2016). The regenerative capacity of EPC can propagate angiogenesis post stroke or in patients with major risk factors for

stroke (Tongers, Roncalli, and Losordo 2010). This is supported by studies on traumatic brain injury where transplanted EPC promoted neurogenesis and amended the endothelial cell integrity (Guo, Deng, and Wei 2017). A higher level of circulating EPC was reported in diabetic patients with cerebral ischaemia when treated with cilostazol (Ueno et al. 2011), which suggests that cilostazol stimulated the endothelial restorative processes. Other studies confirm that treatment with the PDE3 inhibitor cilostazol and the PDE4 inhibitor rolipram had a positive effect on neovascularization in response to ischemic events (Hori et al. 2012; Chen et al. 2018).

The beneficial effects of PDE5 inhibition in stroke is associated with enhanced NO-cGMP signalling, improved cerebral blood perfusion, augmented vascular repair processes, and increased angiogenesis in addition to improved functional recovery in response to ischaemia (Charriaut-Marlangue et al. 2014; Choi, Kim, and Kang 2006; Zhang et al. 2006; Zhang et al. 2003; Zhang et al. 2005; Li et al. 2007). Application of PDE5 inhibitors may be useful both in acute stroke and in secondary stroke prevention since PDE5 inhibitors reduce endothelial cell death and possibly increase angiogenesis, which contributes to neuro-repair, restoration of cerebral blood flow after cerebral ischemia and improves functional recovery after stroke (Menniti et al. 2009; Menniti et al. 2012; Zhang et al. 2006; Zhang et al. 2003). In the recovery phase, post-stroke formation of blood vessels will assist in improving blood supply in the vulnerable hypoperfused brain tissue. VEGF stimulates neurogenesis (Jin et al. 2002) and is shown to possess neuroprotective effect in *in vitro* ischaemia (Jin, Mao, and Greenberg 2000). Treatment of rats with sildenafil resulted in increased plasma nitric oxide levels inducing angiogenesis via synthesis of vascular endothelial growth factor (VEGF) and cGMP (Zhang et al. 2003). Also, cilostazol was reported to stimulate production of VEGF and its receptor, VEGFR, but whether these effects are cAMP- or cGMP-mediated is not fully understood (Omote et al. 2014).

Ischaemic stroke, which induces formation of apoptotic cells, cell debris and increased reactive oxygen species, triggers an inflammatory response in the vessel walls. The neuro-inflammatory process activates microglia and the infiltration of leucocytes from the bloodstream to the site of ischemic injury, which can result in larger infarcts in the post-ischemic tissue (Kawabori and Yenari 2015). Dipyridamole reduced the plasma levels of vWF and CRP in patients with ischemic stroke (Zhao et al. 2006), improved the endothelial reactivity, and inhibited the inflammatory response post stroke (Hallevi, Hazan-Halevy, and Paran 2007; Zhao et al. 2006). Dipyridamole appears to not only prevent platelet aggregation, but it also reduces the impact of ischaemia by reducing the inflammatory response as an add-on effect.

This review assessed the current literature for expression of PDEs in the brain with a focus on PDEs in cerebral microvascular endothelial cells as a therapeutic target to amend endothelial dysfunction in cerebral ischemic stroke. Modulation of both cAMP and cGMP signalling by PDEi possesses potential therapeutic value for BBB repair and protection to alleviate the ischemic stroke injury. Studies which explore the cAMP, cGMP or the cross-talk signalling in endothelial dysfunction are still warranted.

PDEi have been used to treat vascular diseases for decades, but many PDEi are associated with considerable side effects, which leaves a narrow therapeutic window. Having said that, in virtue of the differential tissue and subcellular distribution of the PDE families and their numerous isoforms, PDEs are highly potential drug targets. This diversity allows modulation of more specific molecular and tissue-related signalling pathways, hence drugs with better tolerability and improved clinical efficacy can be developed for treatment. In this review, we found that modulation of either cAMP or cGMP levels with specific PDEi can amend the endothelial function and perhaps improve treatment for ischemic stroke and cerebral endothelial dysfunction at least in *in vitro* models, though human studies are needed to confirm these findings.

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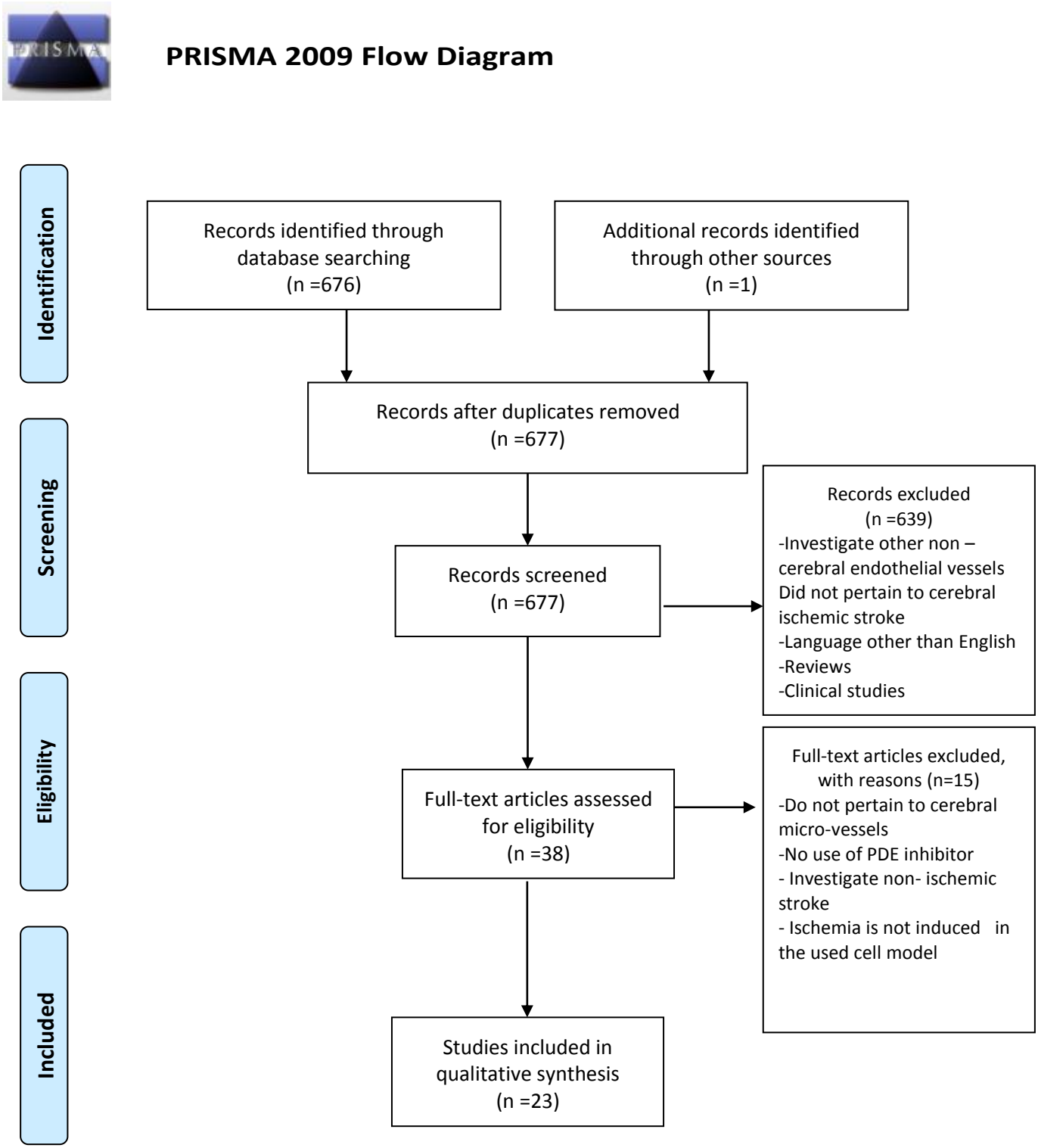

Figure 2. PRISMA flow chart for selection of studies. Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2009 flow diagram. PDE=phosphodiesterase
From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA
Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097. For more information, visit http://www.prisma-statement.org.

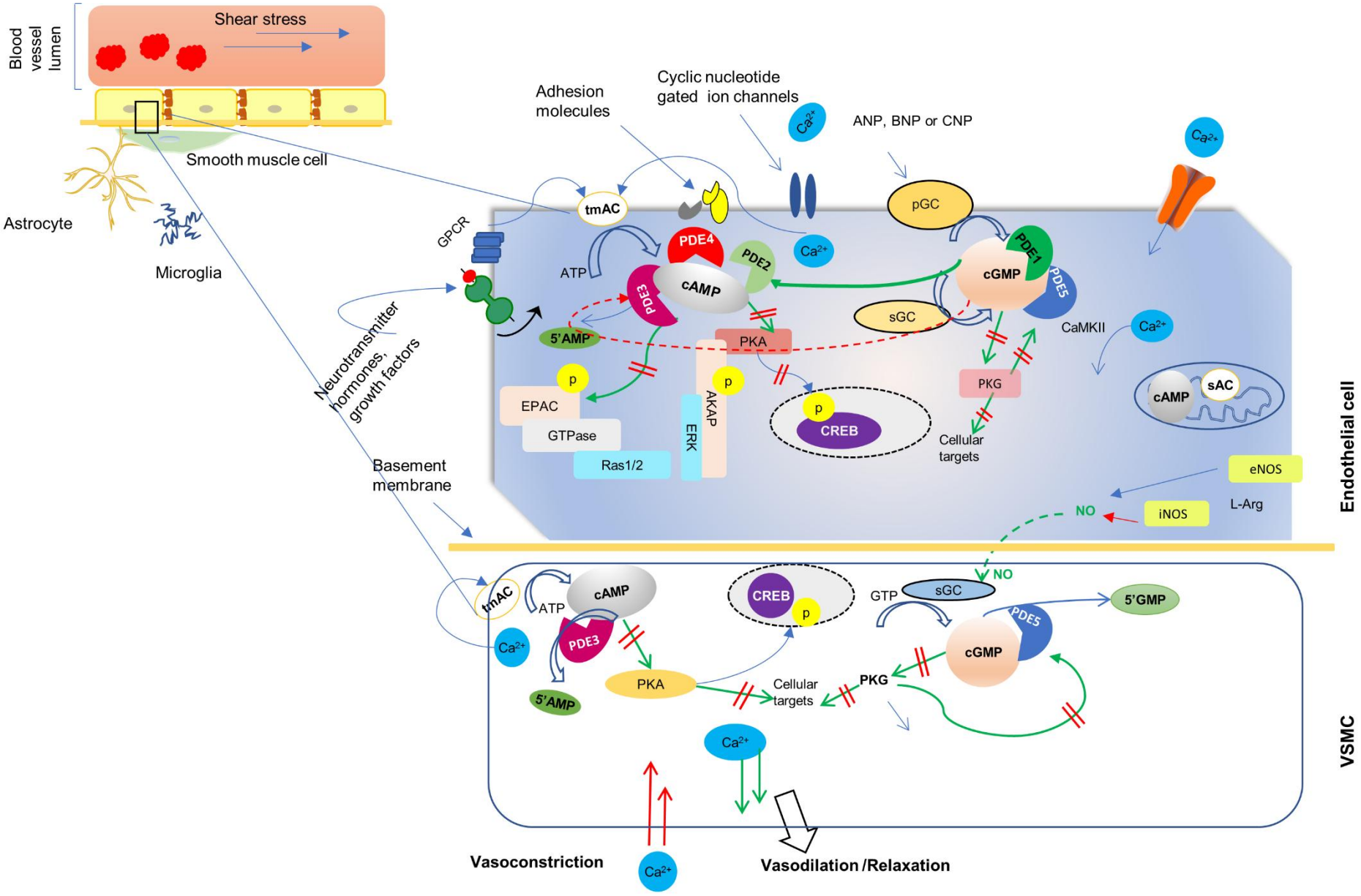
Blood vessel lumen
Shear stress
Smooth muscle cell
Astrocyte
Microglia
Adhesion molecules
Cyclic nucleotide gated ion channels
ANP, BNP or CNP
GPCR
tmAC
ATP
PDE4
PDE2
PDE3
cAMP
5'AMP
Ca2+
pGC
PDE1
PDE5
cGMP
sGC
CaMKII
PKA
EPAC
GTPase
Ras1/2
AKAP
ERK
CREB
PKG
Cellular targets
cAMP
sAC
eNOS
L-Arg
iNOS
NO
Neurotransmitter hormones, growth factors
Basement membrane
Endothelial cell
GTP
5'GMP
VSMC
Vasoconstriction
Vasodilation /Relaxation

Figure 1. A schematic of Endothelial function/dysfunction.

Phosphodiesterase enzymes (PDE) are present in vascular cells, both endothelial and smooth muscle cells. The different PDE families differ in cAMP and cGMP specificity and associate with downstream effector proteins forming signalling domains regulating cyclic nucleotide signalling with a downstream impact on a multitude of biological processes, including endothelial cell signalling.

Signalling domains associated with PDEs also include downstream effector proteins such as A-kinase anchoring proteins (AKAP), exchange protein activated by cAMP (EPAC), protein kinase A (PKA), and protein kinase G (PKG), cyclic nucleotide-gated channels (CNGC), protein phosphatases (PP) and cAMP response element binding protein (CREB). G_s protein-coupled cell-surface receptors respond to extracellular stimulation (hormonal/environmental stimuli and catecholamines) and activate the adenylate cyclase (AC) enzyme, expressed as a transmembrane and a soluble form and initiate the synthesis of cAMP (Inanobe and Kurachi 2014). Guanylate cyclases (GC) synthesize cGMP and exist in two forms: a soluble, which is activated by nitric oxide, and a particulate form, which is activated by natriuretic peptides (ANP, BNP and CNP). cAMP and cGMP exert their function by interaction with several cellular effector proteins such as PKA, PKG, AKAP, and EPAC and regulate downstream signalling pathways, such as the GTPase activated Ras1/2 pathway and the extracellular signal regulated kinase pathway (ERKs). The cyclic nucleotide regulation is tightly regulated by formation of distinct microdomains, containing specific effector proteins, cyclases and PDEs, specific to subcellular location, where they facilitate localized signalling and strictly control activation of off-target effector proteins by the cyclic nucleotide pool.

A healthy endothelium is maintained and stimulated by reactions catalyzed by the blue and green arrows. Two red lines on these reactions indicates inhibition or reduction of the process, which leads to a dysfunctional endothelium. A truncated red arrow is inhibition of PDE3, and a thick green arrow is stimulation of PDE2.

Grey oval structure in the middle of the cell where CREB is located is the nucleus. The color of the endothelium is yellow, and the smooth muscle cell is colored pink. The purple bar between the cells shows the basement membrane. Abbreviations: AKAP, A-kinase anchoring proteins; CNGC, cyclic nucleotide-gated channel; EPAC, exchange protein activated by cAMP; GPCR, G-protein-coupled receptors; PKG, protein kinase G; PP, phosphatase protein; CREB, cAMP response element binding protein; pGC, particulate guanylate cyclase; tmAC, transmembrane adenylate cyclase; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide and CNP, C-type natriuretic peptide.

Table 1. **Expression of PDEs in cerebral vasculature**. Expression of PDE mainly in cerebral microvessels from human, rat and mouse are listed. If location of PDE is not described, it is expressed in the microvessels. hCMEC/D3 is a human cell line and bEND.3 is a mouse cell line and the expression of PDEs may not be like primary cells. R = mRNA transcript; P = protein; A = PDE activity.

PDE form	Human	Protein transcript activity	Rat	Protein transcript activity	Mouse	Protein transcript activity
PDE1	PDE1B1 highest in brain regions: Caudate nucleus, hippocampus, cerebellum (Yu et al. 1997)	R+P+A	PDE1A (He et al. 2011) PDE1B1 (Furuyama et al. 1994) PDE1B1 and PDE1B (He et al. 2011)	R R	PDE1B1 (Yu et al. 1997) (Yan et al. 1994)	R
PDE2	PDE2A2 (10-fold difference: differentially expressed in human cortex and cerebellum) (Lakics, Karran, and Boess 2010) PDE2A in hCMEC/D3 cell line (Schankin et al. 2010) Highest level of PDE2A found in brain (Rosman et al. 1997) PDE2A in hCMEC/D3 cell line (Kalari et al. 2016) PDE2A in granular layer of cerebellum (Sadhu et al. 1999)	R+P R R R R+P	PDE2A2 in cerebral cortical microvessels (Sadhu et al. 1999) PDE2A (Stephenson et al. 2009a) PDE2A (Repaske et al. 1993) PDE2A highly expressed in rat habenula and PDE2A in neurons in dorsal root ganglion (Stephenson et al. 2009a) PDE2 brain (Van Staveren et al. 2003)	R R P R R	PDE2A in endothelial cells of capillaries and veins (Stephenson et al. 2009a) PDE2 expression in mouse endothelial cells from bEND.3 cell line; murine brain endothelium-derived cell line (Vang et al. 2010)	R R
PDE3	PDE3B in hCMEC/D3 cell line (Horai et al. 2013) PDE3B in hCMEC/D3 cell line (Schankin et al. 2010) PDE3 in cerebral arteries (Birk et al. 2004) PDE3A in hCMEC/D3 (Kalari et al. 2016) PDE3A in hCMEC/D3 (Yasmeen et al. 2019)	A+R R+P A R R+P	PDE3A/B (He et al. 2011) PDE3B (Horai et al. 2013) PDE3A and PDE3B in middle cerebral arteries+ basilar artery (Kruse et al. 2006)	A+R R	PDE3B in bEND.3 cell line (Vang et al. 2010)	R
PDE4		R+P	PDE4A, PDE4B, PDE4D (He et	R	PDE4B in mouse endothelial cells, bEND.3	R

	PDE4A-D in hCMEC/D3 cell line (Kalari et al. 2016) PDE4 in cerebral arteries (Birk et al. 2004)	R+A R	al. 2011) PDE4D (He et al. 2012)	R R	cell line (Vang et al. 2010)	
PDE5	PDE5A1 and PDE5A2 in brain (Lin 2004) PDE5A in middle cerebral arteries (Kruuse et al. 2005) PDE5A in hCMEC/D3 cell line (Schankin et al. 2010) (Kalari et al. 2016)	R+P+A R+P R	PDE5A expressed in microvessel and pericytes (He et al. 2012) PDE5 in brain (Van Staveren et al. 2003) PDE5A in arteries and arterioles (He et al. 2011) PDE5A in middle cerebral arteries, basilar artery (Kruse et al. 2006)	R R R+P		
PDE6	PDE6A and PDE6B in hCMEC/D3 cell line (Kalari et al. 2016)	R				
PDE7	PDE7A and PDE7B in hCMEC/D3 cell line (Kalari et al. 2016)	R			PDE7A expression in mouse endothelial cells, bEND.3 cell line (Vang et al. 2010)	R
PDE8	PDE8A and PDE8B in hCMEC/D3 cell line (Kalari et al. 2016)	R			PDE8A expression in mouse endothelial cells, bEND.3 cell line (Vang et al. 2010)	R
PDE9	PDE9A, PDE10A, PDE11a in hCMEC/D3 cell line (Kalari et al. 2016)	R	PDE9A in middle Cerebral arteries, basilar artery, cerebellar cortex (Kruse et al. 2009)	R+P		
PDE10A	PDE10A in hCMEC/D3 cell line (Kalari et al. 2016)	R	PDE10 A in middle Cerebral arteries, basilar artery, cerebellar cortex (Kruse et al. 2009)	R R+P		
PDE11A	PDE11A in hCMEC/D3 cell line (Kalari et al. 2016)	R	PDE11A in middle Cerebral arteries, basilar artery, cerebellar cortex (Kruse et al. 2009)	R+P		

Table 2. Overview of studies with PDE3, PDE4 and PDE5 inhibitors in cerebral ischemic stroke animal/cell models on which the review is based. Studies with patients and non-specific PDEi are not included in the table. Age of the animals is indicated, where the information was available from the original study. Abbreviations used in the table. AGE: advanced glycation end products; CCA: common carotid artery, SHR: spontaneously hypertensive rats; tMCAO: transient middle cerebral artery occlusion; VEGF: vascular endothelial growth factor, HFC: High fat cholesterol diet; CBF: cerebral blood flow; NO: nitric oxide, cGMP: cyclic guanosine mono phosphate, i.p.: intraperitoneal; s.c.: subcutaneous; p.o.: per os; ↑= increase; ↓=decrease and ↔= no change.

Material/species	Stroke model	Treatment/inhibitor	Outcome	Reference
		PDE3 inhibitor		
Triple cell co-culture of rat brain cells (BBB kit ™)	OGD 6 hr	Cilostazol 1 µM 6 hours after OGD	↑Cell tightness	Horai et al.
Triple cell co-culture rat brain cells (Wistar rats) Cells from 3-week-old rats	OGD 3 hr +AGE	Cilostazol 10 µM before and after OGD	↔Cld-5 ↔cell tightness	Takeshita et al.
SHR-SP rats 7-week-old	Risk factor induction: HFC diet	Cilostazol, 100 mg/kg. Daily *i.p.* for 14 days after start of HFC diet +1% NaCl until 10 weeks	↓MMP-9 ↓infarct size ↑ VEGF-R	Omote et al.
CB17/Icr mice 7-week-old	tMCAO 90, 120, 180 and 240 min	Cilostazol 0.3 % in diet For 7 days before induction of ischemia	↓MMP-9 ↓bleeding ↔microvessel density	Kashara et al.
C57BL/6 J mice 10–12 weeks old	tMCAO 45 min and 90 min	Cilostazol 0.3 % in diet For 3 h or 7 days before induction of ischemia	↓bleeding, oedema ↓MMP-9 ↑angiogenesis ↑Neurological outcome ↓Leukocyte migration	Hase et al.
ddY mice 4-week-old	tMCAO 2, 3 and 6 hr	Cilostazol *i.p.* 10 mg/kg After tPA injected 2, 3 and 6 h after ischaemia	↓MMP-9 ↓HT, oedema ↔Cld-5 ↔ microvessel density retained	Ishiguro et al.
ddY mice 4-week-old	tMCAO 3 hr	Cilostazol *i.p.* 1 or 3 mg/kg 21 hours after reperfusion	↑VE-cadherin Cld-5, ZO-1 ↓bleeding	Kitashoji et al.

			↑survival	
C57BL/6J mice 8-10 weeks old	BCCA 15 min	Cilostazol 30 mg/kg p.o. 30 minutes before induction of ischaemia	↓ bleeding ↓Leukocyte migration ↓Platelet adherence	Fukuoka et al.
SHR-SP rats 5-week-old	tMCAO 80 min	5-week treatment with either 0.1 % or 0.3 % cilostazol before induction of ischemia	↓ infarct size ↔ rCBF ↑eNOS phosphorylation	Oyama et al.
		PDE4 inhibitor		
Male Wistar rats Age N.A. Age unknown	tMCAO 2 hr	Rolipram 3mg/kg After ischaemia and treatment continued for 3, 7 or14 days	↓Apoptosis, infarct size ↑Angiogenesis, ↑functional outcome ↑EPC	Hu et al.
Sprague–Dawley rats Age unknown	tMCAO 2 hr	BBB022- PDE4 inhibitor, was infused after 5 hours or 48 hours from ischaemia induction	↑ BBB integrity ↑Functional outcome	Belayev et al.
C57BL/6 mice 6-8 weeks old	tMCAO 2 hr	Rolipram, *i.p.* 2 or 10 mg/kg administered 2 hours after induction of ischaemia	↓ infarct volume, oedema ↓leukocyte migration ↑BBB integrity, tight junction ↓ TNF-α and IL-1β ↓thrombotic vessels	Kraft et al.
		PDE5 inhibitor		
Sprague–Dawley rat pups (P7) Age unknown	Occlusion of right CCA followed by 120 min hypoxia	Sildenafil, i.p. 5 or 10 mg/kg after hypoxia-ischemia start. Animals were euthanized 72 hours and 7 days after hypoxia-ischemia (P14)	↑ motor coordination ↑CBF ↓microglia activation ↓ apoptosis ↑vessel density	Charriaut et al. 2014
Male Wistar rats Age unknown	Embolic MCA	Sildenafil 2mg/kg in water 24 hours after induced	↑ VEGF ↑ angiogenesis	Zhang et al.2003

		stroke and for 6 days		
Male Wistar rats Aged Rats (18 month)	Embolic MCA	Tadalafil, 2 or 10 mg/kg, every other day, starting 24 hours after stroke onset	↑Neurogenesis ↑Angiogenesis ↑cGMP ↑Functional recovery	Zhang et al.2006
SHR-SP rats 6-week-old	High salt diet and fed with inhibitor	PDE5 inhibitor (DA-8159) p.o., 1, 3 and 10 mg/kg, once a day until nearly half of the vehicle-treated animals had died	↑NO ↑cGMP ↑anti-oxidative level ↓cerebral lesion size	Choi et al.
Male Wistar rats Aged Rats 8-12 weeks Young rats 18 month	Embolic MCA	Sildenafil p.o. 3 mg/kg from day 7 and for 7 days after induced ischemia	↑Synaptogenesis ↑Functional recovery ↑cGMP ↑Angiogenesis ↑vessel density	Zhang et al.2005
Male Wistar rats 12-16 weeks	Embolic MCA	Sildenafil s.c. 10 mg/kg, 24 hours after ischaemia and daily for 6 days	↑CBF ↑ vessel density ↑functional recovery	Li et al. 2007