Vascular dysfunction in cerebrovascular disease: mechanisms and therapeutic intervention

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ABSTRACT

The endothelium plays a crucial role in the control of vascular homeostasis through maintaining the synthesis of the vasoprotective molecule NO\(^*\) (nitric oxide). Endothelial dysfunction of cerebral blood vessels, manifested as diminished NO\(^*\) bioavailability, is a common feature of several vascular-related diseases, including hypertension, hypercholesterolaemia, stroke, subarachnoid haemorrhage and Alzheimer’s disease. Over the past several years an enormous amount of research has been devoted to understanding the mechanisms underlying endothelial dysfunction. As such, it has become apparent that, although the diseases associated with impaired NO\(^*\) function are diverse, the underlying causes are similar. For example, compelling evidence indicates that oxidative stress might be an important mechanism of diminished NO\(^*\) signalling in diverse models of cardiovascular ‘high-risk’ states and cerebrovascular disease. Although there are several sources of vascular ROS (reactive oxygen species), the enzyme NADPH oxidase is emerging as a strong candidate for the excessive ROS production that is thought to lead to vascular oxidative stress. The purpose of the present review is to outline some of the mechanisms thought to contribute to endothelial dysfunction in the cerebral vasculature during disease. More specifically, we will highlight current evidence for the involvement of ROS, inflammation, the RhoA/Rho-kinase pathway and amyloid β-peptides. In addition, we will discuss currently available therapies for improving endothelial function and highlight future therapeutic strategies.

INTRODUCTION

The brain has minimal storage of energy sources, making it exceptionally sensitive to interruptions in its blood supply. It is not therefore surprising that the cerebral circulation is a highly specialized vascular bed with a number of unique physiological mechanisms designed to ensure adequate blood flow to the brain over a wide range of external and internal conditions [1,2]. In contrast with vascular beds from the systemic circulation, large arteries are important contributors to cerebral vascular resistance. For example, pressure in the largest intracranial vessels (e.g. the basilar artery) is approx. 80 % of arterial pressure and pressure in pial arteries on the cerebrum [e.g. the MCA (middle cerebral artery)] is approx. 50 % of systemic pressure [3]. In addition, extracranial arteries,
such as the carotid or vertebral arteries, are major sites of resistance to blood flow [3]. As such, modulation of the tone of large intracranial and extracranial arteries, as well as arterioles, plays an important role in regulating CBF (cerebral blood flow). It is now well known that the endothelium plays a major role in maintaining vascular homoeostasis in both cerebral and systemic circulations. In addition, cerebral ECs (endothelial cells), characterized by the presence of unique (in comparison with peripheral ECs) tight cell–cell junctions and a lack of fenestrations, constitute the structural basis of the BBB (blood–brain barrier) [4,5]. The BBB serves the important role of restricting the entry of molecules and immune cells from the systemic circulation into the CNS (central nervous system) [5]. Central to the regulation of cerebrovascular homoeostasis is the endothelium-derived gaseous signalling molecule NO$, which regulates VSM (vascular smooth muscle) tone and inhibits platelet aggregation, leucocyte adhesion and VSM cell growth [2]. The vasculature is constitutively exposed to NO$ from endothelial and neuronal isoforms of NOS (NO$ synthase), eNOS and nNOS respectively. Another source of NO$ is iNOS (inducible NOS), which is not normally expressed but can be induced in response to a variety of inflammatory stimuli. With respect to vascular tone, NO$ stimulates sGC (soluble guanylate cyclase) in VSM resulting in an increase in the concentration of cGMP and subsequent relaxation [2]. The term endothelial dysfunction has been used to refer to several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth and dysregulation of vascular structure. However, more commonly this term is used to refer to an impairment of endothelium-dependent vasorelaxation caused by diminished NO$ bioactivity. Endothelial dysfunction in the cerebral circulation is associated with a number of vascular-related diseases including hypertension [6–10], hypercholesterolaemia [11–13], diabetes [14,15], stroke [16–19] and Alzheimer’s disease [20–23]. Moreover, endothelial dysfunction is associated with increased risk of acute clinical events, such as stroke [24,25], and may contribute to cognitive decline [22]. In light of the many consequences of compromised NO$ signalling it is important to define the pathological mechanisms involved. The study of ROS (reactive oxygen species) and oxidative stress is currently an area of intense research focus, particularly in relation to endothelial dysfunction. Indeed, accumulating evidence implicates oxidative stress as an important underlying cause of cerebral endothelial dysfunction in a number of disorders. Oxidative stress does not act alone; in fact, evidence suggests that other mechanisms, such as inflammation, RhoA/Rho-kinase and Aβ$ (amyloid β-peptides), are also likely to contribute either directly or by promoting oxidative stress. In the present review, we will first focus on some of mechanisms of endothelial dysfunction in the cerebral circulation and, secondly, we will discuss therapeutic approaches for restoring endothelial function.

**MECHANISMS OF ENDOTHELIAL DYSFUNCTION**

**Vascular oxidative stress**

ROS include the $O_2^-$ (superoxide anion) and $OH^*$ (hydroxyl) free radicals, and non-radicals, such as $H_2O_2$. In addition to ROS, a number of RNS (reactive nitrogen species) are produced within vascular cells including $ONOO^-$ (peroxynitrite) (Figure 1). The parent ROS molecule $O_2^-$ can be generated by several enzyme systems, including the mitochondrial electron-transport chain, COXs (cyclo-oxygenases), lipoxygenases, cytochrome P450 reductases, xanthine oxidase, NOS and NADPH oxidases. In the normal physiological state, ROS levels within vascular cells are tightly controlled by SOD1, SOD2 and SOD3 (superoxide dismutase 1, 2 and 3), catalase and GPX (glutathione peroxidase) (Figure 1). Under these conditions, ROS are believed to serve as important signalling molecules for the regulation of normal vascular function (for reviews see [1,26]). However, under conditions of enhanced ROS generation and/or impaired ROS metabolism, oxidative stress can develop. As mentioned, compelling evidence indicates that oxidative stress is an important underlying mechanism of endothelial dysfunction.

**Superoxide**

When $O_2^-$ generation is enhanced or its metabolism is decreased in the vascular wall, the major consequence is the reaction of $O_2^-$ with NO$, which is three times faster than the dismutation of $O_2^-$ by SOD [27]. Clinical and experimental evidence suggests that oxidative inactivation of NO$ is an important underlying mechanism of impaired NO$-dependent responses associated with cerebrovascular disease and aging. Indeed, $O_2^-$ scavengers improve extra- and intracranial cerebrovascular NO$-dependent responses in aged animals [28–30] and in models of hypertension [7–9,31,32], hypercholesterolaemia [12,13,33,34], hyperhomocysteinemia [35,36], Alzheimer’s disease (see the Amyloid β-peptides section), SAH (subarachnoid haemorrhage) [37], diabetes [38–41] and stroke [42]. Furthermore, a study of SOD-deficient mice reported a potential role for $O_2^-$ in promoting hypertrophy of cerebral first-order pial arterioles [43], possibly through the inactivation of NO$ or through direct activation of signalling cascades involved in growth of VSM cells [44]. In addition to its effect on NO$ bioavailability, $O_2^-$ might impair NO$-mediated signalling by decreasing the expression and activity of sGC [45,46]. In addition, $O_2^-$ has been reported to cause apoptosis of cerebral
Figure 1  Schematic illustration of the generation and interactions of vascular ROS and NO•

O2•− is generated from the one electron reduction of O2 by various oxidases and is metabolized by SODs. There are three isoforms of SOD, namely cytosolic CuZn-SOD (SOD1), mitochondrial Mn-SOD (SOD2) and extracellular CuZn-SOD (SOD3). SOD metabolizes O2•− to H2O2, which in turn is metabolized to water by catalase or GPXs. In the normal physiological state, ROS levels within vascular cells are tightly controlled by these antioxidant enzymes. However, under conditions of enhanced ROS and/or impaired ROS metabolism, oxidative stress can develop. Oxidative stress is believed to be an important underlying mechanism of diminished NO• function. For example, O2•− can readily react with NO• to form ONOO−, which in turn can oxidize the eNOS cofactor BH4, resulting in ‘eNOS uncoupling’ and further formation of O2•−. ONOO− can also oxidize sGC to its ferric Fe3+-bound form, rendering it unresponsive to NO•. H2O2 can react with Fe2+ to form the highly reactive OH• radical. In addition, H2O2 may activate NADPH oxidases and modulate eNOS expression. BH3, trihydrobiopterin; BH2, 7,8-dihydrobiopterin.

Hydrogen peroxide

A large body of evidence indicates that H2O2 may represent an important vasoactive substance capable of regulating cerebrovascular tone during (patho)physiological conditions. Indeed, H2O2 is often reported to be a dilator, but sometimes is a constrictor in cerebral blood vessels [1,55]. In the cerebral circulation, H2O2 has been implicated in mediating flow-dependent vasodilator responses [56], in modulating contractile responses to AngII (angiotensin II) [55,57], and in mediating dilator responses to bradykinin [58] and AA (arachidonic acid) [59]. H2O2 acutely stimulates NO• production by eNOS in non-cerebral arteries [60] and up-regulates eNOS expression in vivo and in vitro [61,62]. Taken together these findings point towards a beneficial role for H2O2 in modulating cerebrovascular function under some conditions [1]. Consistent with this hypothesis is the finding that H2O2-mediated vasodilatation of the rat basilar artery by NADPH (the substrate for NADPH oxidase) is augmented during experimental hypertension [63]. However, the potential long-term consequences of enhanced levels of H2O2 and downstream ROS for vascular function should not be ignored. Indeed, evidence suggests that H2O2 may impair cerebrovascular function through its conversion into microvascular ECs [47] and elicit either relaxation or contraction of cerebral arteries independent of its effect on NO• [1].

Peroxynitrite

ONOO−, the reaction product of O2•− and NO•, has been reported to cause nitration of cerebral intracortical arterioles and capillaries [10], and attenuate NO•-dependent cerebrovascular vasodilator responses [10,42,48]. Studies of non-cerebral arteries indicate that ONOO− can oxidize BH4 (tetrahydrobiopterin; a cofactor for eNOS) [49–51] and the zinc–thiolate complex in eNOS [50], leading to eNOS uncoupling (see the Other sources of ROS section) and subsequent O2•− production (Figure 1). However, it remains to be determined whether ONOO− similarly promotes eNOS uncoupling in the cerebral vasculature. ONOO− might also impair NO• signalling by oxidizing the haem of sGC to its ferric NO•-insensitive state [52] (Figure 1). Another possible effect of ONOO− might include damage to DNA, leading to the activation of the DNA repair enzyme PARP [poly(ADP-ribose) polymerase] and vascular dysfunction [28,53]. Indeed, ONOO−-induced PARP activation has been implicated in cerebrovascular dysfunction associated with aging [34].
Sources of ROS

NADPH oxidases

NADPH oxidases are now believed to be a major source of ROS in both cerebral and systemic vasculatures (for reviews see [1,68,69]). These enzymes are similar, but not identical, in structure to the phagocytic NADPH oxidase, comprising two membrane-bound subunits [Nox (the catalytic subunit) and p22phox], up to three cytoplasmic subunits (p47phox/NoxO1 and p67phox/NoxA1) and a small G-protein (Rac1/Rac2) [1]. To date, several NADPH oxidase isoforms have been identified of which Nox1-, Nox2-, Nox4- and Nox5-containing NADPH oxidases are known to be expressed in vascular cells [69]. Interestingly, it has become evident that the activity of NADPH oxidases is greater in cerebral arteries, such as the MCA and basilar artery, than in a range of systemic arteries from several animal species under physiological conditions [57,70]. Furthermore, higher cerebrovascular Nox2 and/or Nox4 activity may, in part, account for these regional differences [57,70]. Importantly, augmented NADPH oxidase activity and expression in the cerebral vasculature has been described in aging [29,30] and in disease models, such as hypertension [9,10,32,63], hypercholesterolaemia [12], Alzheimer’s disease (see the Amyloid β–peptides section), SAH [37,71,72], diabetes [73] and stroke [42,74–76]. Thus one would predict that such changes would contribute to increased vascular ROS levels and hence endothelial dysfunction. Indeed, studies have found that inhibitors of NADPH oxidase improve endothelial function in several disease models [9,37,73,75,77,78]. In mouse MCAs, cerebral arterioles and capillaries, Nox2 is primarily localized to ECs [32,55]. In cultured basilar artery ECs, Nox2 mRNA is normally expressed at lower levels than either Nox1 or Nox4 mRNA [79]. In addition, Nox2 appears not to be constitutively active under basal conditions [47]. However, studies using Nox2-deficient mice reveal that this isoform might play a central role in endothelial dysfunction of the cerebral circulation [9,10,22,30,32,78]. For example, mice lacking Nox2 do not exhibit cerebrovascular oxidative stress and are protected from the alterations in cerebrovascular endothelial function induced by AngII [8,9,32]. The role(s) of other NADPH oxidase isoforms in cerebrovascular dysfunction is less clear. However, the observations that: (i) Nox4 is constitutively active in cerebral artery/arteriole ECs under physiological conditions [47]; (ii) in the rat, Nox4 protein expression is higher in the basilar arteries and MCAs compared with a range of systemic arteries [57]; (iii) Nox4 protein expression in the rat basilar artery is augmented during hypertension [63]; and (iv) basilar artery Nox1 and Nox4 protein expression are higher in male compared with female rats [80], suggest that these NADPH oxidase isoforms may be functionally important under (patho)physiological conditions. To the best of our knowledge we are unaware of any studies testing the importance of Nox1 in endothelial dysfunction of cerebral arteries. However, a recent study reported that Nox4 is a major source of O2− that causes apoptosis of cerebral microvascular ECs exposed to the pro-inflammatory cytokine TNFα (tumour necrosis factor α) [47]. Currently it is unclear whether Nox5 is functionally important in the cerebral circulation. Investigation of the role of this isoform is hampered by the fact that rodents do not express the gene for Nox5.

Other sources of ROS

Evidence to date suggests that NADPH oxidases may be a major source of pathological ROS; however, the potential for other contributing sources should not be overlooked. Mitochondria generate O2− as a by-product of oxidative phosphorylation and might be an important source of O2− in the cerebral circulation, given cerebrovascular ECs contain more mitochondria than ECs in other vascular beds [81]. A role for mitochondria-derived ROS in mediating relaxation of cerebral pial VSM cells has been demonstrated [82,83]. In contrast, evidence indicates that changes in the mitochondrial redox status and ROS production might have a negative impact on vascular function. For example, NO•-dependent relaxation responses are impaired in cerebral arterioles of heterozygous SOD2 (also known as mitochondrial Mn-SOD)-deficient mice, an effect that is normalized by a ROS scavenger [84]. Recent evidence suggests that cross-talk might exist between NADPH oxidase- and mitochondrial-derived ROS, at least in response to AngII [85,86]. More specifically, it has been reported that AngII causes mitochondrial dysfunction in cultured non-cerebral ECs via activation of NADPH oxidase [85]. Moreover, AngII-induced up-regulation of NADPH oxidase subunits is attenuated in mice overexpressing the mitochondrial antioxidant Trx2 (thioredoxin 2) [86]. Another enzymatic pathway capable of generating O2− in the cerebral circulation involves xanthine oxidase, an
enzyme which catalyses the breakdown of hypoxanthine into uric acid. Future studies are needed to establish whether such interactions are also important in cerebral arteries. A wealth of epidemiological data shows that elevated uric acid is associated with an increased risk of vascular events in the post-stroke period [87]. In a small clinical trial, allopurinol, a specific inhibitor of xanthine oxidase, improved cerebrovascular endothelial function in patients with Type 2 diabetes [88]. In contrast, a larger clinical trial found no benefit of allopurinol on cerebrovascular endothelial function in patients following stroke [89]. COXs have repeatedly been shown to be an important source of ROS in the brain and cerebral circulation [1]. For example, increases in $O_2^\cdot$ and changes in cerebrovascular tone in response to AA are dependent on the activity of COXs [90]. Under certain circumstances, eNOS can generate $O_2^\cdot$ instead of NO; this is called ‘uncoupling’ of eNOS. Although eNOS uncoupling has been attributed to diverse mechanisms in experimental conditions, a decrease in BH4 bioavailability is thought to be the most important mechanism [91]. In non-cerebral arteries, evidence suggests that uncoupled eNOS might contribute to vascular oxidative stress in a number of diseases including hypertension and atherosclerosis [91]. In contrast, much less is known regarding the contribution of uncoupled eNOS to pathological ROS in cerebral vessels. Recently, it was reported that BH4 treatment and NOS inhibition normalized $O_2^\cdot$ production and increased CBF following hypoxic/ischaemic brain injury in newborns, raising the possibility that uncoupled eNOS (or other NOS isoforms) contributes to vascular dysfunction following stroke [92]. Interestingly, a previous study suggested that eNOS is uncoupled under normal conditions in cerebral arteries [93]. However, the overall importance of this latter finding and mechanism is difficult to assess given several other studies show that eNOS generates NO* and not ROS under physiological conditions [94]. Evidence suggests that expression of iNOS might play an important role in the development of endothelial dysfunction of the carotid artery and cerebral arterioles during diabetes [14,95]. Furthermore, impaired NO*-dependent relaxation has been reported to occur in human, canine and rabbit cerebral arteries following gene transfer of iNOS [15,96,97]. Potential mechanisms by which up-regulation of iNOS may impair endothelial function probably involves oxidative stress. Excessive amounts of NO* generated by iNOS may react with $O_2^\cdot$ to form ONOO* in vivo. In addition, uncoupling of iNOS and subsequent $O_2^\cdot$ production can occur when the availability of substrate or cofactors is limited [98]. Indeed, $O_2^\cdot$ levels are elevated in the canine basilar artery after gene transfer of iNOS [97]. Furthermore, sepiapterin, a precursor of BH4 biosynthesis, improves endothelial function of iNOS-transfected rabbit carotid arteries [15]. Clearly, further work is needed to more fully define the relative importance of NADPH oxidases, mitochondria, xanthine oxidase, COXs, uncoupled NOS and other enzymes as sources of (patho)physiological ROS in the cerebral circulation.

**ROS and BBB dysfunction**

As mentioned above, the cerebral endothelium constitutes the structural basis of the BBB, which plays a critical role in regulating the homeostasis of the CNS [5]. Accumulating experimental and clinical evidence indicates that BBB dysfunction is associated with a number of vascular-related diseases including ischaemic stroke, diabetes and Alzheimer’s disease [5]. The pathways thought to initiate BBB dysfunction associated with such diseases are numerous; however, all appear to converge on the same point: oxidative stress [99]. In both *in vitro* and *in vivo* models, $O_2^\cdot$ or other ROS increase BBB permeability [94]. It has been reported that both inhibition of NADPH oxidases or genetic deletion of Nox2 ameliorates BBB dysfunction after experimental stroke [75]. ROS are likely to trigger a number of downstream pathways that directly mediate BBB dysfunction, such as oxidative damage, tight junction modification, activation of matrix metalloproteinases and components of the immune system [99].

**Inflammation**

Recent evidence points to a role for inflammation in vascular dysfunction associated with a number of disease states. Studies of systemic arteries indicate that components of the immune system are activated in the vessel wall in a number of cardiovascular diseases. Furthermore, complex interactions between immune cells (e.g. T-cells, macrophages and dendritic cells), vascular cells and pro-inflammatory cytokines [e.g. TNFα, IL (interleukin)-1, IL-6 and interferon-γ] are now thought to contribute to functional and structural vascular changes associated with cardiovascular disease [100–102]. Similarly, activation of the immune system has been implicated in cerebrovascular dysfunction associated with cerebral ischaemia and reperfusion [103]; however, its contribution to dysfunction of the cerebral circulation in other disease states remains to be fully tested. Evidence suggests that hypercholesterolaemia causes an increase in leucocyte and platelet adherence, and rolling in the cerebral microvasculature, in a Nox2-NADPH-oxidase-dependent manner [104]. However, intracranial cerebral arteries (e.g. the basilar artery and MCA) and arterioles actually appear to be less susceptible to developing atherosclerotic lesions than extracranial arteries [12,105,106]. Interestingly, TNFα has been shown to activate cerebrovascular NADPH oxidase, resulting in the generation of $H_2O_2$ and subsequent cerebral vasodilatation [107]. That finding suggests that TNFα could be helpful rather than harmful in supporting CBF during disease. Yet, recent evidence suggests that
Figure 2  Proposed interactions between Rho-kinase, ROS and endothelial NO$^\bullet$

It is well established that RhoA can directly inhibit eNOS, and subsequent NO$^\bullet$ production, via interference with the eNOS mRNA stability. More recently, the potential of Rho-kinase to suppress PI3K activity within the endothelium, and subsequent NO$^\bullet$ production, has been described. Normally, stimulation of PI3K results in phosphorylation and activation of the protein kinase Akt, leading to eNOS activation. The prospect of negative regulation of PI3K by Rho-kinase raises the possibility that endothelial Rho-kinase (as opposed to its VSM form) also modulates endothelial function. Specifically, this interaction could lead to decreased NO$^\bullet$ bioavailability and subsequent endothelial dysfunction. Although several studies have also suggested that Rho-kinase might interact with ROS, such as O$_2^-$, the precise nature of this interaction remains unclear and whether this interaction occurs within the endothelium of cerebral vessels is yet to be determined. Nevertheless, it is likely that such an interaction would exacerbate endothelial dysfunction during disease by further reducing NO$^\bullet$ bioavailability.

TNFα can cause apoptosis of cerebral microvascular ECs via augmented O$_2^-$ production by Nox4-NADPH-oxidase [47]. Moreover, TNFα and other inflammatory stimuli can up-regulate the expression of iNOS in vascular cells [2], which, as discussed above, has been suggested to cause cerebral endothelial dysfunction in at least some cardiovascular disease states. Clearly, future work is needed to elucidate the overall effect(s) of TNFα in cerebrovascular function. Besides TNFα, recent evidence suggests that other cytokines may modulate cerebral endothelial function. Specifically, it has been reported that carotid arteries from IL-6-deficient mice are protected against the endothelial dysfunction produced by AngII both in vivo and in vitro, suggesting a role for IL-6, probably within the vascular wall, in mediating the effects of AngII on endothelial function [8]. In addition, AngII failed to increase vascular O$_2^-$ levels in vitro in vessels from either IL-6- or Nox2-deficient mice [8]. Thus IL-6 might be a critical link in the NADPH-oxidase-mediated impairment of NO$^\bullet$-mediated vascular signalling associated with hypertension [8]. A more recent study found that IL-10 deficiency augments carotid artery O$_2^-$ levels and endothelial dysfunction in response to AngII both in vivo and in vitro [7], suggesting that the anti-inflammatory cytokine IL-10 may be a key mediator of vascular protection during hypertension and in other disease states where AngII plays a major role [7].

RhoA/Rho-kinase pathway

The development of relatively selective pharmacological inhibitors of RhoA/Rho-kinase, such as Y-27632, fasudil (also known as HA1077) and C3 exoenzyme, has considerably advanced our understanding of the role of this pathway in regulating cerebrovascular function. Indeed, the RhoA/Rho-kinase pathway has been implicated in regulating basal cerebrovascular tone [108–113] and in mediating vasoconstrictor responses to pathologically important molecules, such as AngII [31], serotonin [114], endothelin-1 [115], UTP [116,117] and oxyhaemoglobin [118]. Furthermore, evidence suggests that augmented RhoA/Rho-kinase signalling in vascular cells contributes to cerebrovascular dysfunction in disease states, such as stroke, SAH, hypertension and diabetes (for reviews see [119–122]).

Endothelial RhoA/Rho-kinase and NO$^\bullet$

To date, the contribution of RhoA/Rho-kinase to the dysregulation of cerebrovascular function has largely been considered with respect to its increased signalling activity in VSM. However, recent studies (albeit largely in non-cerebral vessels) raise the possibility that augmented RhoA/Rho-kinase signalling in ECs may also contribute to cerebrovascular dysfunction during disease. For instance, it has been demonstrated in human ECs that RhoA can negatively regulate eNOS protein expression by destabilizing its mRNA [123]. Moreover, Rho-kinase itself has also been reported to negatively regulate eNOS phosphorylation via inhibitory effects on PI3K (phosphoinositide 3-kinase) and Akt [124], which are known to contribute to NO$^\bullet$ production [125] (Figure 2). Consistent with this notion, fasudil augments eNOS mRNA and protein levels in cultured ECs [126]. It has also been demonstrated that Rho-kinase might attenuate NO$^\bullet$ production in ECs via inhibition of PI3K activity [127] (Figure 2). That study reported that treatment of human ECs with fasudil can lead to a rapid increase in Akt phosphorylation and NO$^\bullet$ production, an effect blocked by PI3K inhibition [127]. The first
functional evidence of this phenomenon has since been demonstrated in intact arteries, where agonist-induced contractile responses of rat aorta were inhibited by Y-27632 in an endothelium-, PI3K- and NOS-dependent manner [128]. Moreover, in healthy humans, increases in forearm blood flow in response to fasudil are markedly attenuated when co-infused with the NOS inhibitor l-NMMA (Nω-monomethyl-l-arginine), providing the first evidence that RhoA/Rho-kinase signalling may play an important role in regulating eNOS in human arteries in vivo [129]. Consistent with this, fasudil has been reported to augment NO* bioavailability and improve endothelial function of the brachial artery in human subjects with coronary artery disease [130]. Moreover, a significant correlation has been reported between impaired NO*-dependent vasodilator responses and augmen ted vasodilator responses to fasudil in cigarette smokers [130]. Collectively, these findings raise the possibility that endothelial RhoA/Rho-kinase signalling might tonically inhibit NO* production even in healthy arteries and that this effect might be augmented in disease.

It actually remains unclear whether RhoA/Rho-kinase normally modulates endothelial NO* production in the cerebral circulation. However, several lines of evidence suggest a role for augmented endothelial RhoA/Rho-kinase signalling in ischaemic stroke [119]. First, Rho-kinase activity is increased in the endothelium of rat penumbral microvessels in the early period after focal cerebral ischaemia [131]. Secondly, in mice, Y-27632 and fasudil acutely improve CBF during the ischaemic core and penumbra [126,132]. Moreover, this improvement in CBF is absent in eNOS-deficient mice [132], thus suggesting that augmented Rho-kinase activity in the endothelium might contribute to decreased NO* bioavailability during disease. Consistent with this hypothesis, a recent study reported that fasudil prevented impaired NO*-dependent relaxation of cerebral arterioles from rats exposed to cigarette smoke [133]. Examination of the pleiotropic effects of statins also supports the notion that endothelial RhoA/Rho-kinase contributes to decreased NO* bioavailability in cerebrovascular disease (see the Therapeutic interventions for endothelial dysfunction section).

RhoA/Rho-kinase and NADPH oxidases
Evidence also suggests that an interaction between RhoA/Rho-kinase and NADPH oxidases might exist (Figure 2). Indeed, RhoA/Rho-kinase can be activated by ROS or AngII [31,75,134–136], leading to VSM contraction. Furthermore, Rho-kinase inhibition has been reported to suppress AngII-stimulated O2*—production by endothelial NADPH oxidases and to decrease the expression of Nox1, Nox2, Nox4 and p22(phox) [137]. It remains to be established whether a similar relationship exists between endothelial RhoA/Rho-kinase and NADPH oxidase in the cerebral circulation. However, it is conceivable that augmented endothelial RhoA/Rho-kinase signalling might also contribute to cerebrovascular endothelial dysfunction by promoting oxidative stress.

**Amyloid β-peptide**
Aβ is cleaved from the APP (amyloid precursor protein) by two aspartyl proteases, named β-secre tase and γ-secretase. Cleavage by these proteases yields a family of Aβs, with a 40-amino-acid species (Aβ1−40) and a 42-amino-acid species (Aβ1−42) predominating. An imbalance between the production and removal of Aβs from the brain can lead to their deposition in the brain parenchyma as senile plaques, as seen in Alzheimer’s disease, or in the cerebral vasculature, as seen in CAA (cerebral amyloid angiopathy). Vascular Aβ deposition is a relatively common pathology associated with aging and is prevalent in Alzheimer’s disease [138,139]. Furthermore, CAA is a major cause of brain haemorrhage and is now recognized as a potential cause of cerebral ischaemia and cognitive impairment, independent of stroke [140]. Vascular Aβ deposition is associated with impaired NO*-dependent relaxation responses of the MCA and arterioles in experimental models [20–22]. Moreover, evidence links elevations in soluble Aβ with impaired cerebrovascular function even in the absence of vascular deposition. For instance, exogenous Aβs constrict the cerebral arteries (basilar artery and MCA) of humans and rodents [141,142]. Furthermore, some [78,143–145], but not all [146], studies have reported that APP transgenic mice exhibit cerebrovascular dysfunction, such as impaired NO*-dependent relaxation responses, reductions in CBF, abnormal cerebrovascular autoregulation and impaired functional hyperaemia at an early age, when amyloid plaques and behavioural deficits are not yet present. Furthermore, application of exogenous Aβ1−40 to mouse cerebral arterioles results in similar cerebrovascular alterations to those seen in APP transgenic mice [78,145,147,148]. Thus disruptions in cerebrovascular function by soluble Aβ might be an early event in Aβ-related diseases [149]. This hypothesis is supported by clinical studies showing that changes in cerebral perfusion precede the onset of dementia in Alzheimer’s patients [150].

**Aβs and oxidative stress**
The precise mechanisms by which Aβs impair endothelial function have not been fully elucidated. In the scenario where deposition occurs, Aβ may present a mechanical obstacle to vasodilatation, rendering the vessel wall relatively rigid [21]. Additionally, reductions in the number of VSM cells or their reactivity may similarly impair vasodilatation responses [146]. Notwithstanding this, other evidence suggests that oxidative stress plays an important role in mediating the effects...
of $A\beta$ on cerebrovascular function. First, exogenous treatment of cerebral microvessels with $A\beta_{1-40}$ augments ROS production [148]. Secondly, APP transgenic mice exhibit signs of vascular oxidative stress [22,148,151]. Thirdly, overexpression of SOD or treatment with ROS scavengers improves vascular function in young and aged APP transgenic mice [20,22,147,148,152]. One of the mechanisms involved in the dysfunction is likely to be related to a reduction in NO* bioavailability. Indeed, exogenous $A\beta_{1-40}$ attenuates the increase in CBF produced by a NO* donor and NO inhibition blocks the effect of exogenous $A\beta_{1-40}$ on cerebrovascular function [78].

$A\beta$s and NADPH oxidases
Several lines of evidence indicate that NADPH oxidase is the enzymatic source of the ROS responsible for the vascular effects of $A\beta$s. The NADPH oxidase cytosolic subunits, p47$^{phox}$ and p67$^{phox}$, are translocated to the membrane in brain tissue from Alzheimer’s disease patients [153], suggesting that NADPH oxidase is activated during Alzheimer’s disease. Park et al. [78] found that either inhibition of NADPH oxidase or genetic deletion of Nox2 counteracts the oxidative stress and endothelial dysfunction induced by exogenous $A\beta_{1-40}$. Moreover, genetic deletion of Nox2 abrogates cerebrovascular dysfunction in young APP transgenic mice [78]. Similarly, either NADPH oxidase inhibition or Nox2 deletion restored endothelial function in cerebral arteries from aged APP transgenic mice, suggesting that in more advanced stages of pathology NADPH-oxidase-derived ROS remain the major initiator of endothelial dysfunction [154]. Nevertheless, the specific molecular cascades that lead to $A\beta$-induced activation of vascular NADPH oxidase remain to be fully understood.

EDHF (endothelium-derived hyperpolarizing factor) and endothelial function
EDHF is an important vasodilator mechanism in some cerebral blood vessels. EDHF relaxes VSM through hyperpolarization and this involves activation of $K^+$ channels [155]. There is evidence that, as in systemic arteries, the functional importance of EDHF in endothelium-dependent relaxation appears to become more prominent (with the role of NO* diminishing) in smaller cerebral arteries/arterioles [19,156] Evidence suggests that EDHF acts as a compensatory vasodilator under conditions when NO* function is compromised. For example, Cipolla et al. [19] reported that basal NO* production by rat cerebral arterioles is diminished following ischaemia and reperfusion, whereas EDHF-mediated responses are preserved. The functional roles of EDHF remain to be fully determined; however, these findings raise the possibility that EDHF may play a beneficial role in maintaining CBF in diseases associated with impaired NO* function [19].

THERAPEUTIC INTERVENTIONS FOR ENDOTHELIAL DYSFUNCTION

Clinical drugs
HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors (statins)
The beneficial vascular effects of statins are now known to extend beyond their cholesterol-lowering activity. One such effect of statins is an improvement in vascular NO* bioavailability and hence endothelial function [16,151,157,158]. Sander et al. [159] reported an improvement in cerebral vasoreactivity in healthy adults after short-term statin treatment. Similarly, statin therapy improves cerebrovascular endothelial function in humans with lacunar stroke, hypertension and hypercholesterolaemia [18,157]. Moreover, improvement in CBF and reduction in cerebral infarct volume (after MCA occlusion) by statins is eNOS-dependent in normocholesterolaemic mice [160,161]. Evidence suggests that statins may, in part, augment NO* bioavailability via inhibition of RhoA (Figure 3). Specifically, statins prevent the formation of isoprenoid intermediates required for the geranylgeranylation of RhoA, which is a prerequisite for its activation. Thus by preventing this process, statins inactivate RhoA, leading to increased Akt phosphorylation [162], eNOS expression and activity, and increased endothelial NO* production [121,163]. Isoprenylation is also required for activation of Rac1 (an NADPH-oxidase-associated G-protein) therefore it is likely that statins may additionally improve endothelial function by decreasing oxidative stress. Accordingly, statin therapy has been reported to inhibit cerebrovascular $O_2^-$ production by NADPH oxidase in a model of diabetes [158] and decrease cerebrovascular oxidative stress in aged APP transgenic mice [151]. It is also of note that statins may decrease oxidative stress by limiting eNOS uncoupling through an increase in endothelial BH$_4$ levels [164].

PPAR$\gamma$ (peroxisome-proliferator-activated receptor $\gamma$) agonists
PPAR$\gamma$ is a ligand-activated transcription factor targeted by the TZDs (thiazolidinediones), a class of anti-diabetic drugs. TZDs, such as rosiglitazone and pioglitazone, are now well known to improve insulin sensitivity, and lower blood pressure and the risk of recurrent stroke in patients with Type 2 diabetes [165,166]. It is of interest that emerging evidence suggests that PPAR$\gamma$ might be a critical regulator of cerebral vascular function. Indeed, either global (via knockout or genetic expression of a dominant-negative variant) or EC-specific (via the
Figure 3  Current and potential therapeutic strategies for the treatment of cerebral endothelial dysfunction

The signalling pathways thought to be involved in the development of cerebral endothelial dysfunction can be pharmacologically targeted at several levels, either by drugs already in clinical use or by more novel compounds. For instance, statins, widely used for treating hypercholesterolaemia, have repeatedly been shown to increase NO• levels. This is thought to occur through inhibition of the small G-proteins Rho and/or Rac, resulting in increased eNOS mRNA stability, and potential interference with NADPH oxidase function respectively, the latter resulting in decreased generation of ROS. PPARγ agonists, such as TZDs, are currently in use for the treatment of diabetes; however, emerging evidence suggests that they may be a novel therapeutic strategy to improve NO• bioavailability and limit oxidative stress in the cerebral vasculature. In light of evidence supporting NADPH oxidases as a primary source of pathological ROS, one might predict that interventions that inhibit NADPH oxidase (e.g. apocynin) will improve cerebral endothelial function. Rho-kinase inhibitors, such as fasudil, already in clinical use for post-stroke treatment, also increase NO• levels, possibly via indirect activation of PI3K. As well as these established therapies, there is still scope for further development of novel and perhaps even more selective therapeutic agents. The potential value of compounds that target both the reduced and oxidized forms of sGC, ROS scavengers and mimetics of endogenous SOD may all offer much promise in improving cerebral endothelial function during disease.

dominant-negative variant) PPARγ interference in mice causes vascular hypertrophy and remodelling of mouse cerebral arterioles [167], as well as impaired basilar artery NO•-dependent relaxation responses [168]. Moreover, cerebral artery NO•-dependent responses are restored by an O2− scavenger [168]. Thus PPARγ might normally serve a protective role in regulating NO• bioavailability in the cerebral circulation via inhibiting oxidative stress (Figure 3). Consistent with this, PPARγ agonists decrease the expression of NADPH oxidase and AT1 receptors (AngII type 1 receptors), but increase SOD1 expression, in non-cerebral vascular cells [169,170]. Thus it might be anticipated that activation of PPARγ with synthetic agonists would have beneficial effects on cerebrovascular endothelial function. Indeed, PPARγ agonists decrease O2− levels and improve endothelial function in the carotid artery of hypertensive mice and rats [171,172]. Similarly, a PPARγ agonist has recently been reported to normalize NO•-dependent responses of the MCA in APP transgenic mice [20]. Clearly, PPARγ agonists offer much promise in improving cerebral endothelial function; however, it is important to note that the clinical safety of currently used TZDs, in particular rosiglitazone, has been challenged [173]. Consequently, there has been an increased focus on identifying and testing novel compounds that target PPARγ [174].

Antioxidant strategies

As discussed in the present review, oxidative stress represents an important underlying cause of endothelial dysfunction. Therefore it is not surprising that there has been enormous interest in developing strategies that target ROS. However, it is important to recognize that relatively low levels of ROS serve a physiological role in regulating cerebrovascular function. Thus future therapeutic approaches that alleviate the burden of oxidative stress need to be delicately balanced to ensure that important cellular processes remain intact.

Antioxidants

Numerous experimental studies and small clinical trials have reported a beneficial effect of short- and long-term administration of antioxidants, such as ascorbic acid (vitamin C) and tocopherol (vitamin E), on endothelial function in disease states associated with oxidative stress. However, the results of numerous large-scale
clinical trials of antioxidant supplementation in vascular disease have been disappointing (for a review see [175]). Indeed, virtually all of these clinical trials failed to demonstrate significant beneficial effects of antioxidants on cardiovascular end points. The reasons for this may relate to the prescription of a suboptimal dose. Indeed, it is known that supraphysiological concentrations of vitamins C and E are required to compete with the reaction of $\text{O}_2^-$ and NO$^\cdot$. Alternatively, it is possible that orally administered vitamins may be inaccessible to the source (i.e. cells within the vessel wall) of ROS, that intervention occurs too late in the progression of disease, and/or that there are potential pro-oxidant properties of vitamins. Either way, ‘classical’ antioxidant therapy would now seem to be of limited clinical use in improving endothelial function in disease states associated with oxidative stress. Another approach to removing $\text{O}_2^-$ from the vessel wall could be to enhance its metabolism by SOD. The therapeutic potential of administering native SOD is limited by its low stability and inability to permeate cells. However, modification of SOD by adding PEG [poly(ethylene glycol)] to its structure improves its stability and ability to enter cells. Recently, much effort has been made to develop small cell-permeant molecules that mimic native SOD (Figure 3). Examples include manganese porphyrins, such as MnTMPyP [Mn(III)tetakis (1-methyl-4-pyridyl)porphyrin] and MnTBAP [Mn(III)tetakis (4-benzoic acid)porphyrin], and M40403 [Mn bis(cyclohexyl pyridine) macrocyclic complex]. Experimental evidence suggests some potential therapeutic benefit of SOD mimetics in reducing oxidative stress; however, further studies are necessary to fully establish their real therapeutic efficacy [1,69]. Over the past 5 years, the therapeutic potential of ebselen (an ONOO$^-$ scavenger) and edavarone (an $\text{O}_2^-$ scavenger), and the radical-trapping agent disufenton sodium (NXY-059), for the treatment of stroke have been tested. Although some clinical benefit was found with both ebselen and edavarone, no such benefit has been demonstrated with NXY-059 [176].

Antioxidant gene therapy
Targeting antioxidant enzymes using gene therapy might be another more direct approach to increasing antioxidant capacity in the vessel wall [177]. To date, a number of antioxidant genes have been successfully delivered via gene therapy, including SOD2, SOD3, catalase, GPX and haem oxygenase-1 [177]. Experimental studies (mostly in non-cerebral arteries) have shown that antioxidant gene therapy can improve endothelial function in a number of disease models, such as hypertension [178,179], hypercholesterolaemia [180] and diabetes [181]. Another novel gene therapy approach to augment antioxidant defences might be to use transcription factors. For example, a study has found that gene transfer of the transcription factor Nrf2 (nuclear factor-E2-related factor-2), which binds to the so-called antioxidant-response element (a common regulatory element found in the 5'-flanking regions of antioxidant enzymes), in a rabbit balloon injury model reduced oxidative stress in the aorta [182]. However, gene therapy for the treatment of cerebrovascular disease is still in its infancy. To date, gene transfer of SOD3 has been shown to reduce early [183], but not delayed/persistent [184], cerebral vasospasm in models of SAH. Furthermore, gene transfer of GPX has been used successfully for protection against experimental stroke [185]. Future basic and preclinical studies are necessary to realize the potential of this therapeutic strategy for improving cerebrovascular endothelial function in disease. However, the translation of gene therapy into the clinic might be hampered by several factors, such as the limited efficacy of current delivery methods and vectors, and the potential for vectors to evoke an immune response [177].

Selective inhibition of NADPH oxidases
In light of evidence supporting NADPH oxidases as a primary source of pathological ROS, one might predict that interventions that inhibit NADPH oxidases will improve endothelial function. Over the past decade, several inhibitors of NADPH oxidase have been developed, including diphenyleneiodonium, apocynin and gp91ds-tat. However, the majority of these have a number of non-specific effects that will prevent or limit their clinical usefulness [69]. Furthermore, all appear to inhibit both vascular and immune cell isoforms of NADPH oxidase thus, if given systemically for more than a few days, would be expected to compromise immune function. The mechanism(s) of action, non-specific effects and therapeutic potential of currently available pharmacological inhibitors of NADPH oxidases has recently been comprehensively reviewed [69]. More work is still needed to identify novel vascular-isoform-specific inhibitors of NADPH oxidase and to test their therapeutic potential for improving endothelial function in humans.

Inhibition of Rho-kinase
In addition to ameliorating the effects of RhoA/Rho-kinase signalling in VSM, inhibition of Rho-kinase might have the added benefit of improving NO$^\cdot$ bioavailability. A number of reasonably selective pharmacological inhibitors of Rho-kinase have been developed (for reviews see [120,121,186]) and have been used extensively in experimental studies. However, somewhat surprisingly, there has so far been a paucity of clinical trials testing the therapeutic potential of these compounds, especially in relation to diseases associated with the cerebral circulation. Indeed, fasudil is currently the only Rho-kinase inhibitor that is available for clinical use, largely due to its good safety profile. Fasudil is used clinically in Japan for the treatment of cerebral
vasospasm after SAH [187] and has shown efficacy in the treatment of experimental stroke; however, only one Phase III trial has tested its efficacy in humans [188]. That trial found that treatment of patients with fasudil 48 h following acute ischaemic stroke improved clinical outcome [188]. However, the significance of these findings is somewhat unclear as the trial was performed in a relatively small number of patients, used limited neurological assessment methods and only included patients with atherothrombotic or lacunar strokes [188].

Nitroxyl (HNO) donors and NO*-independent sGC activators

The clinical efficacy of traditional NO* donors is limited by their susceptibility to tolerance development, decreased effectiveness under oxidative stress and cytotoxic effects. HNO donors and NO*-independent soluble sGC activators are rapidly emerging as novel pharmacological agents with several therapeutic advantages over traditional NO* donors [189,190]. Like its redox sibling NO*, HNO serves as a potent vasorelaxant, lowers blood pressure and inhibits platelet aggregation, predominantly via sGC activation and an elevation in cGMP [190]. Of importance, HNO is resistant to both scavenging by O₂⁻ and tolerance development [190]. Thus its chemistry and vasodilatory profile make HNO (and its donors) a potentially useful pharmacological tool to treat vascular diseases in which endothelial dysfunction and oxidative stress occur, but this remains to be tested. Additionally, a novel series of NO*-independent activators of sGC (i.e. BAY 58-2667 and HMR1766) have been developed. These compounds serve as vasorelaxants, inhibit platelet aggregation and decrease blood pressure under normotensive and hypertensive conditions [52,189]. Importantly, BAY 58-2667 can potently bind and activate oxidized and haem-free sGC, leading to enzyme activation and cGMP production [191] (Figure 3). These unique properties of NO*-independent sGC activators may allow them to selectively target the diseased vasculature [52].

SUMMARY

Endothelial dysfunction in the cerebral circulation appears to be a common feature in a number of vascular-related diseases. The impact of endothelial dysfunction in the brain is particularly far reaching because NO* plays an integral role in maintaining vascular homeostasis through the regulation of VSM tone and structure, platelet aggregation and immune surveillance. From the evidence outlined in the present review, it is apparent that, although the diseases associated with endothelial dysfunction are diverse, the underlying causes are actually very similar. In particular, there is compelling evidence that oxidative mechanisms may be a common pathway leading to compromised NO* signalling in several cardiovascular 'high-risk' states and cerebrovascular disease. Moreover, evidence suggests that AngII, components of the immune system, the RhoA/Rho-kinase pathway and Aβs may mediate at least some of their deleterious effects on endothelial function by promoting oxidative stress. Although NADPH oxidases are emerging as an important source of ROS, contributing to vascular oxidative stress, future work is needed to evaluate the importance of other sources of ROS, such as the mitochondria, xanthine oxidase, COX and uncoupled eNOS. As outlined in the present review, a number of therapeutic approaches to improve endothelial function have been considered. In particular, there has been an increased focus on developing therapies that alleviate vascular oxidative stress. A key goal of future work will be to elucidate the extent to which current and novel therapeutic approaches aimed at improving endothelial function provide real benefits with respect to reducing clinical vascular events.

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REFERENCES


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