[Frontiers in Bioscience 16, 1733-1745, January 1, 2011]

Oxidative stress and endothelial dysfunction in cerebrovascular disease

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1. ABSTRACT

Maintenance of vascular tone by the endothelium involves the production of endothelium-derived nitric oxide (NO). NO, produced from endothelial nitric oxide synthase diffuses to the underlying smooth muscle to stimulate soluble guanylate cyclase, resulting in increased cyclic GMP levels, and subsequent smooth muscle relaxation and blood vessel dilatation. Endothelial dysfunction, manifested as diminished NO bioavailability, is a common feature of a number of vascular-related diseases.. Oxidative stress can be defined as an imbalance between reactive oxygen species (ROS) production and/or impaired ROS metabolism that favours them being present in excess of physiological levels. Oxidative stress can negatively impact many cell types, including in the vasculature. There is now a wealth of evidence suggesting that oxidative stress is a major cause of endothelial dysfunction in the cerebral circulation. This review will summarize disease models in which both oxidative stress and endothelial dysfunction occur in the cerebral circulation, namely hypertension involving angiotensin II (Ang II), diabetes, subarachnoid hemorrhage, stroke and Alzheimer's disease. Molecular mechanisms by which oxidative stress occurs, (eg increased NADPH-oxidase activity) will also be discussed.

2. INTRODUCTION

Reactive oxygen species (ROS) include the superoxide and hydroxyl free radicals, and non-radicals, such as hydrogen peroxide. In addition to ROS, a number of reactive nitrogen species (RNS) are produced within vascular cells including peroxynitrite. The parent ROS molecule superoxide can be generated by several enzyme systems, including NADPH-oxidases, cyclooxygenases (COXs), the mitochondrial electron transport chain, xanthine oxidase and uncoupled endothelial nitric oxide synthase (eNOS) (1) (Figure 1). Antioxidant defense mechanisms include the superoxide dismutases (SODs), of which there are three isoforms expressed in the vasculature: cytosolic or copper-zinc SOD (CuZnSOD or SOD-1), manganese SOD (MnSOD or SOD-2) localized in mitochondria, and an extracellular form of CuZn-SOD (ECSOD or SOD-3) (2). Glutathione peroxidases are expressed in blood vessels and are also an important antioxidant defense mechanism (3).

Generally speaking, physiological levels of ROS serve as mediators and modulators of cell signaling, and are important for maintaining vascular homeostasis (4). However, under conditions of enhanced ROS generation

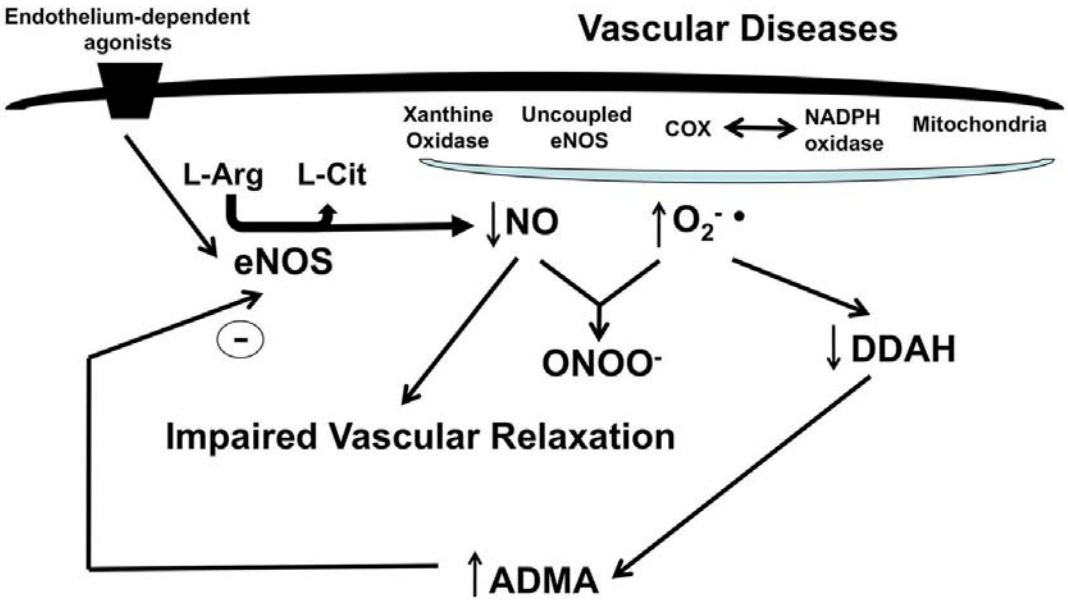

Figure 1. Endothelium-dependent agonists can stimulate the production of eNOS-derived nitric oxide (NO) in the endothelium, which then diffuses to the underlying smooth muscle to elicit vascular relaxation. During cerebral vascular disease states, increases in reactive oxygen species (especially superoxide, O_2^-) are generated by several sources (e.g. NADPH-oxidases, cyclooxygenases [COXs] and the mitochondria). COX activity may also be necessary to facilitate Ang II-induced increases in ROS via NADPH oxidase (see ref 41). Superoxide reacts extremely efficiently with NO to form the reactive nitrogen species, peroxynitrite ($ONOO^-$), leading to a decrease in NO bioavailability and impaired vascular relaxation. Oxidative stress can also reduce dimethylarginine dimethylamine hydrolase (DDAH) activity, leading to increased asymmetric dimethylarginine (ADMA) levels, decreased eNOS activity and impaired vascular relaxation (see text for details).

and/or impaired ROS metabolism, oxidative stress can develop. Evidence indicates that oxidative stress may contribute to the initiation and development of a number of disease states, including (but certainly not limited to) hypertension and stroke (5-8).

The endothelium is a single cell layer that lines the luminal surface of blood vessels and which provides a protective barrier between tissues and the circulating blood, as well as being involved in the regulation of vascular tone and vascular structure. An essential component of the maintenance of vascular tone by the endothelium involves the production of endothelial-derived nitric oxide (NO). NO, produced from eNOS, diffuses to the underlying smooth muscle to stimulate soluble guanylate cyclase (sGC), resulting in increased levels of cyclic GMP, and subsequent smooth muscle relaxation and blood vessel dilatation (9), (Figure). The term endothelial dysfunction has been used to refer to several pathological conditions, including altered anti-coagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular structure (10). However, more commonly this term is used to refer to an impairment of endothelium-dependent vasorelaxation caused by diminished NO· bioactivity. It is this particular aspect of endothelial dysfunction that will be the focus of this review. Endothelial dysfunction in the cerebral circulation is associated with a number of vascular-related diseases including hypertension, diabetes and stroke. Moreover, endothelial dysfunction is associated with increased risk of acute clinical events, such as stroke (11, 12) and may contribute to cognitive decline (13). Over the past several years an enormous amount of research has been devoted to understanding the mechanisms underlying endothelial dysfunction. As such, compelling evidence implicates oxidative stress as an important underlying cause of cerebral endothelial dysfunction in a number of disorders.

The aim of this review is to summarize key findings where oxidative stress and endothelial dysfunction occur, namely hypertension involving angiotensin II (Ang II), diabetes, subarachnoid hemorrhage (SAH), stroke and Alzheimer's disease. In particular, this review will focus on experimental evidence for the occurrence of oxidative stress and endothelial dysfunction in the cerebral circulation, as well as the enzymatic sources of ROS implicated in the development of oxidative stress. The potential protective role of SOD isoforms and dimethylarginine dimethylamine hydrolase (DDAH) in protecting against endothelial dysfunction will also be discussed.

3. OXIDATIVE STRESS AND CEREBRAL ENDOTHELIAL DYSFUNCTION IN ANIMAL MODELS OF CARDIOVASCULAR DISEASE

As mentioned, oxidative stress is believed to be a major cause of endothelial dysfunction in the cerebral circulation (10, 14). When superoxide generation is enhanced or its metabolism is decreased in the vascular

Table 1. Summary of experimental models with oxidative stress and endothelial dysfunction in the cerebral circulation

Disease	Animal Model	EDR Reversed by ROS Scavenger	References
Hypertension (Increased Ang II)	-Systemic Ang II infusion in mice -R^+/A^+ mice -Acute Ang II treatment	↓ EDR reversed by tiron, tempol, MnTBAP,PEG-SOD	(40-48)
Diabetes	-Mouse (db/db; tallyHo) -Rat (STZ-i.v.) -Rat (OLETF) -Obese Zucker rat	↓ EDR reversed by tempol and PEG-SOD	(19, 20, 22-25)
Aging	12-24 month old mice	↓ EDR reversed by tempol, MnTBAP, AT_1R-deficiency	(92, 105, 106)
Hypercholesterolemia	$ApoE^{-/-}$mouse	↓ EDR reversed by tiron, tempol, MnTBAP,PEG-SOD	(107-109)
Hyperhomocysteinemia	*Cbs*$^{+/-}$ mice fed a high methionine diet	↓ EDR reversed by tiron	(110)
Alcohol	Chronic (3 month) alcohol diet	↓ EDR	(111)
Smoking	-i.v. infusion of nicotine for 30 mins -nicotine via osmotic minipump -cigarette smoke inhalation	↓ EDR reversed by tiron, tempol, MnTBAP,PEG-SOD	(112-115)
SAH	-Rat double hemorrhage model -Rat single hemorrhage model -Endovascular perforation of rat ACA	↓ Luminal diameter	(52-54)
Hypoxia	Vessels exposed to no-oxygen conditions	↓ EDR reversed by tempol	(62)
PPARγ interference	-P465L mutation -Endothelium-specific PPARgamma mutation + high fat	↓ EDR reversed by tempol	(116-118)
Alzheimer's disease	-Topical treatment of mouse cerebral vessels with Aβ 1-40 -APP overexpressing mice	↓ EDR reversed by tempol, SOD, catalase, N-acetyl-cysteine, pioglitazone	(13, 26, 28-35, 119)
SOD deficiency	$CuZnSOD^{-/-}$ $MnSOD^{+/-}$ $ECSOD^{-/-}$	↓ EDR reversed by tempol	(46, 47, 92-94)
Stroke	SHRSP	↓ EDR reversed by losartan and pioglitazone	(120-124)
Cerebral Ischemia	MCAO/reperfusion	↓ EDR	(60, 64, 66-68, 125-128)

wall, the major consequence is its reaction with NO, resulting in reduced NO bioavailability both under basal conditions and in response to endothelium-dependent agonists which stimulate NO production. A loss of bioavailable NO results in less NO acting on the underlying smooth muscle, and thus impaired vasodilatation. In addition, the reaction of superoxide with NO leads to the formation of the highly reactive RNS peroxynitrite. Peroxynitrite can further impair NO signaling and enhance oxidative stress by oxidation of tetrahydrobiopterin (cofactor for eNOS) (15), causing eNOS to produce superoxide rather than NO (ie. eNOS uncoupling), and by activating poly (ADP-ribose) polymerase (PARP) (16). A survey of the literature reveals that there is a wealth of biochemical, functional and molecular evidence that link oxidative stress and cerebral endothelial dysfunction in a number of disease models, and these will be discussed below (Table 1).

3.1. Diabetes

Type II diabetes is a major risk factor for stroke as well as for Alzheimer's disease (17, 18). In *db/db* mice (a genetic model of type II diabetes), superoxide levels (measured by hydroethidine based confocal microscopy) in cerebral arterioles were elevated when compared with levels in control mice (19). Similarly, in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, another model of type II diabetes, basilar artery superoxide levels (measured by amount of nitro blue tetrazolium reduced) were increased when compared with control rats (20). In the obese Zucker rat, a model of metabolic syndrome which involves hyperglycaemia, hyperinsulinemia, insulin-resistance, hypertriglyceridemia and hypercholesterolemia (21, 22), cerebral artery superoxide levels (measured using dihydroethidene fluorescence microscopy) were augmented when compared to the lean Zucker rat (22). Also, in streptozotocin-treated rats (a model of type 1 diabetes) superoxide levels in the parietal cortex were found to be increased when compared with non-diabetic rats (23, 24). Moreover, this increase in superoxide was attenuated by the superoxide spin trap agent tempol (24). Taken together, these findings provide evidence that superoxide levels are elevated in both the brain and cerebral vasculature in several experimental models of diabetes. Evidence indicates that cerebral endothelial dysfunction occurs in all these aforementioned models of diabetes (19, 20, 22, 23), and in tallyHo mice (another genetic model of type II diabetes) (25), probably as a result of oxidative inactivation of NO. For example, in cerebral arterioles from db/db and tallyHo mice, impaired NO-dependent relaxation responses were reversed by acute application of polyethylene glycol (PEG)-SOD (19, 25). Similarly, in OLETF rats, impaired NO-dependent relaxations of the basilar artery were significantly improved by tempol or PEG-SOD (20). Also, in the obese Zucker rat, impaired NO-dependent relaxation responses of the middle cerebral artery (MCA) were partially reversed by tempol (22). Thus, these data suggest that inactivation of NO by superoxide might be an important underlying mechanism of impaired NO-dependent responses in the cerebral circulation during diabetes.

3.2. Alzheimer's disease

Alzheimer's disease is a complex clinical condition that is thought to be largely neurodegenerative,

however recent evidence indicates there is a vascular component to this disease, and it is that aspect of Alzheimer's disease pathology we wish to discuss here. Alzheimer's disease progression is perhaps mediated, in part, by the overproduction of the amyloid beta (Abeta) peptide, a cleavage product of the amyloid precursor protein (APP) (26). An experimental model of Alzheimer's disease, which has been widely studied in recent years, are mice expressing human APP carrying an Alzheimer's-associated mutation. These mice exhibit elevated brain levels of Abeta, and develop cognitive, neuropathological and cerebral metabolic alterations that resemble those of Alzheimer's disease (27, 28). Cerebral vascular deposition of Abeta (cerebral amyloid angiopathy) and also elevations in soluble Abeta are associated with impaired cerebral endothelial function. For example, in APP transgenic mice, NO-dependent increases in cerebral blood flow (CBF) (13, 28, 29), and NO-dependent relaxation responses of the isolated MCA (30-32) were impaired compared to responses in wild-type mice. Furthermore, application of exogenous Abeta 1-40 (one of the predominant Abeta species) to mouse cerebral arterioles (26, 33) and the rat basilar artery (34) resulted in similar cerebral vascular alterations to those seen in APP transgenic mice. The precise mechanisms by which Abeta impairs endothelial function have not been fully elucidated, however, several lines of evidence suggest a role for oxidative stress. In APP transgenic mice and in wild-type mice where the cerebral cortex was treated with Abeta 1-40 increases in ROS levels were observed in neurons and endothelial cells (13,26). Moreover, this increase in ROS was inhibited by the ROS scavenger MnTBAP (13). Cerebral vascular nitrosative stress is also evident in the cerebral circulation of APP transgenic mice. For example, increased 3-nitrotyrosine immunoreactivity, indicative of nitrosative stress, was found in both small cortical microvessels (30) and endothelial cells of pial arterioles (35) of APP transgenic mice compared with wild-type controls**.** Also, impaired NO-dependent responses were not apparent in APP mice overexpressing SOD1 (29), and when either SOD (29) or MnTBAP (13) were applied to the cerebral cortex. Endothelial dysfunction of the isolated MCA from APP mice was acutely reversed by SOD and catalase, (30). *In vivo* treatment with tempol and N-acetyl-cysteine (a precursor for cysteine, which is important in glutathione synthesis. Glutathione plays an important role in antioxidant defense (36)) also reversed the endothelial dysfunction (31). Moreover, the effect of exogenous Abeta1-40 on cerebral vascular NO-dependent responses was reversed by treatment with both SOD and MnTBAP (33), and prevented by a NOS inhibitor (26). More recently, the PPAR gamma (peroxisome proliferator-activated receptor gamma) agonist pioglitazone was also found to improve endothelial function in APP transgenic mice, an effect the authors attributed to increased NO bioavailability due to suppression of NADPH-oxidase activity and/or enhanced SOD1 activity by pioglitazone (31). Taken together, these data suggest a role for augmented superoxide and hydrogen peroxide production by NADPH-oxidases (see Section 4.0: Sources of Oxidative Stress and Cerebral Endothelial Dysfunction) in the impairment of endothelial function associated with augmented levels of Aβ.

3.3. Hypertension Involving Elevated Ang II

The renin-angiotensin system and its main effector, Ang II, underlie many of the changes in vascular structure and function that occur in several forms of hypertension (8, 37). Indeed, pharmacological inhibitors of the renin-angiotensin system are widely used in the clinic for the treatment of hypertension (38). Hypertension has profound effects on the cerebral circulation and is a major risk factor for stroke (39). A wealth of evidence indicates that Ang II increases ROS production in the cerebral circulation. Several studies have reported that acute intravenous infusion of mice with Ang II increased blood pressure and ROS production by cerebral blood vessels (40-44). Furthermore, the increase in cerebral vascular ROS production was prevented by treatment with MnTBAP (44). Acute intravenous infusion of Ang II has been reported to increase 3-nitrotyrosine immunoreactivity in mouse cerebral vascular endothelial cells, an effect that was prevented by a peroxynitrite scavenger and NOS inhibitor, and also in Nox2-deficient mice (41). Thus, these findings suggest that Ang II increases peroxynitrite formation in the cerebral vasculature largely via the reaction of Nox2-NADPH-oxidase-derived superoxide with NO.

Acute intravenous administration of Ang II has also been reported to impair NO-dependent increases in CBF (43, 44)), an effect that was reversed by MnTBAP and the angiotensin type 1 (AT_1) receptor antagonist losartan (44). In mice overexpressing human renin and angiotensinogen (a genetic model of chronic hypertension), endothelial dysfunction of the basilar artery was completely reversed by PEG-SOD (45). Similarly, Ang II-induced endothelial dysfunction in cerebral arterioles of ECSOD deficient mice was reversed by tempol (46). Importantly, systemic administration of a non-pressor dose of Ang II also caused endothelial dysfunction in the basilar artery (47). Endothelial dysfunction in response to Ang II was reversed by tempol (47). Moreover, topical application of Ang II to cerebral arterioles *in vivo* caused impaired NO-dependent responses that could be prevented by the superoxide scavenger tiron (48). Taken together, these data suggest that Ang II causes endothelial dysfunction in the cerebral circulation by activating AT_1 receptors on the vessel wall leading to an increase in superoxide production, and subsequent oxidative inactivation of NO.

3.4. Subarachnoid hemorrhage (SAH)

SAH is a unique disorder and a major clinical problem that most commonly occurs when an aneurysm in a cerebral artery ruptures, leading to bleeding and clot formation. SAH results in death or severe disability of 50-70% of victims and is the cause of up to 10% of all strokes (49). Cerebral vasospasm leading to brain ischaemia is a critical clinical complication that occurs after SAH. Indeed, decreased NO bioactivity and oxidative stress are both thought to be involved in cerebral vasospasm (50, 51). Several studies have shown that SAH is associated with elevated levels of superoxide in cerebral arteries. For

example, increased superoxide production was reported in the MCA from rats injected twice with autologous blood to induce SAH (52). Cerebral vascular superoxide production by NADPH-oxidase was ~3-fold higher in rats following subarachnoid injection of autologous blood compared with controls (53). Similarly, in another model of SAH (endovascular perforation of the right anterior cerebral artery), superoxide levels and NADPH-oxidase activity were increased by ~2-fold compared with controls (54). Thus, these studies would suggest that superoxide production by NADPH oxidases is augmented in SAH, and may be associated with endothelial dysfunction and reduced diameter of cerebral arteries following SAH (52, 54-56).

3.5. Experimental Cerebral Ischaemia and Reperfusion

There is now a considerable body of evidence indicating that oxidative stress in the brain is a fundamental mechanism of neuronal damage following cerebral ischaemia and reperfusion (4, 57). During the early stages of post-ischaemic cerebral reperfusion, the function and integrity of cerebral arteries are critical to support CBF and thus minimize further neuronal damage (58). As in systemic vascular beds, reperfusion (up to 6 hours) after partial or complete cerebral ischaemia is known to result in excessive production of cerebral vascular ROS, including hydrogen peroxide, hydroxyl, peroxynitrite and predominantly superoxide (59-61). Similarly, superoxide production by rat basilar arteries is augmented in response to hypoxia/reoxygenation *in vitro* (62). In addition, Gursoy-Ozdemir *et al* found that 3-nitrotyrosine immunoreactivity is increased in mouse cerebral microvessels and capillaries of the ischaemic hemisphere during the early reperfusion (up to 6 hours) period following transient middle cerebral artery occlusion (MCAO) (63). Of potential importance, evidence now suggests that augmented cerebral vascular superoxide production persists for several days after the initial ischaemic insult. Indeed, we have found that superoxide production by NADPH-oxidases is elevated in rat arteries from the ischaemic hemisphere for up to 3 days after mild cerebral ischaemia (64).

An increase in the production of NO in the brain has been demonstrated during ischaemia (65). Endothelial NO production could conceivably improve CBF following ischaemia and reperfusion, however, a concomitant surge in superoxide production is likely to lead to the formation of peroxynitrite and hence a loss of bioavailable NO. Indeed, as mentioned there is evidence of oxidative/nitrosative stress in cerebral blood vessels after ischaemia and reperfusion. Moreover, several studies have reported that NO-dependent responses of cerebral arteries are impaired in models of ischemia and reperfusion (60, 62, 66-68). Furthermore, there is some evidence that superoxide and peroxynitrite scavengers can improve cerebral vascular NO-dependent responses following cerebral ischaemia and reperfusion (60, 62, 68). The enzymatic source of cerebral vascular superoxide during ischaemia and reperfusion remains to be fully elucidated, however, evidence suggests a possible role for NADPH-oxidases (see section 4: Sources of Oxidative Stress and Cerebral Endothelial Dysfunction).

3.6. Clinical stroke

eNOS oxidizes its substrate L-arginine to form L-citrulline and NO (Figure 1). In this manner, L-arginine is thought to act on the vascular endothelium to result in increased production of NO. Therefore, using cerebrovascular reactivity to L-arginine as a measure of cerebral endothelial function, it was found that cerebral endothelial function in stroke patients was impaired when compared with healthy controls (69, 70). Similarly, cerebrovascular reactivity to L-arginine was impaired in ischemic versus non-ischemic hemispheres of stroke patients (71). Furthermore, flow-mediated dilatation of the brachial artery was also impaired, suggesting that both cerebral and peripheral endothelial function is impaired in stroke patients (69, 72). It is currently unclear whether the endothelial dysfunction found in stroke patients is related to oxidative stress. However such a mechanism appears likely given the wealth of evidence from animal models of stroke, and other diseases that predispose to stroke, linking oxidative stress with endothelial dysfunction.

4. SOURCES OF OXIDATIVE STRESS AND CEREBRAL ENDOTHELIAL DYSFUNCTION

4.1. NADPH-oxidase

NADPH-oxidases comprise two membrane-bound subunits, Nox (catalytic subunit) and p22phox, up to three regulatory cytoplasmic subunits (p47phox/NoxO1 and p67phox/NoxA1) and a small G-protein (Rac1/Rac2) (4, 73). 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 (4, 73). As has been alluded to above and is further discussed below, there is now a substantial body of evidence (including pharmacological, biochemical and molecular) to suggest that NADPH-oxidases are likely contributors to the development of oxidative stress and endothelial dysfunction in the cerebral circulation in a number of disorders. Pharmacological evidence comes largely from studies using apocynin (an NADPH oxidase inhibitor), and while the selectivity of apocynin for NADPH oxidase has recently been questioned (74), evidence using other approaches also supports a role for NADPH oxidase in these disorders. Of potential importance, NADPH-oxidase activity and function is reportedly greater in cerebral versus systemic arteries under physiological conditions in at least four animal species (75, 76). As such, the cerebral vasculature may be relatively more susceptible to the development of oxidative stress.

4.1.1. Diabetes

In OLETF rats, impaired NO-dependent relaxation of the basilar artery could be reversed by the NADPH-oxidase inhibitor apocynin (20). Protein expression of Nox2 was also found to be increased in basilar arteries of OLETF versus control rats (20). Thus, this study would suggest a role for increased Nox2-NADPH-oxidase expression and hence activity as a mediator of oxidative stress and endothelial dysfunction during type II diabetes (20).

4.1.2. Alzheimer's disease

Several lines of evidence indicate that NADPH-oxidase is the likely enzymatic source of the ROS responsible for the detrimental effects of Abeta on cerebrovascular function. Park *et al.* found that either inhibition of NADPH-oxidase activity with gp91ds-tat (a peptide that inhibits $p47^{phox}$ association with Nox2 (77)) or genetic deletion of Nox2 (26), counteracts the oxidative stress and endothelial dysfunction induced by exogenous Aβ 1-40. Moreover, genetic deletion of Nox2 abrogates cerebrovascular dysfunction in young APP transgenic mice (26). Similarly, either NADPH-oxidase inhibition or Nox2 deletion restored endothelial function in cerebral arteries from aged APP mice, suggesting that in more advanced stages of pathology, NADPH-oxidase-derived ROS remain an important initiator of oxidative stress and subsequent endothelial dysfunction (78).

4.1.3. Hypertension Involving Ang II

It has been demonstrated that increases in ROS and endothelial dysfunction following Ang II treatment do not occur in cerebral arterioles treated with gp91ds-tat, suggesting a role for NADPH-oxidase (44). These findings were extended and similar findings observed in mice genetically deficient in Nox2 (40, 41, 44). Indeed, the endothelial dysfunction that is normally observed in the cerebral circulation of wild-type mice following Ang II treatment was absent in Nox2-deficient mice despite the fact that Ang II still elevated blood pressure in these genetically modified mice (44, 79). Thus, the increase in blood pressure caused by Ang II is unlikely to contribute to the development of endothelial dysfunction in the cerebral circulation, but instead points to a direct effect of Ang II on vascular NADPH-oxidase. Furthermore, Ang II had only minimal effect on relaxation responses to acetylcholine in Nox1-deficient mice, suggesting that Nox1 may also contribute to Ang II-induced cerebral endothelial dysfunction (79). Another study reported that superoxide generation by cerebral arteries in response to Ang II is partially Nox1-dependent (80). Overall, these data support the concept that cerebral vascular oxidative stress and endothelial dysfunction in response to increased levels of Ang II is largely dependent on Nox2-NADPH-oxidase, with some role for Nox1-NADPH oxidase.

4.1.4. Subarachnoid hemorrhage

The activity of vascular NADPH-oxidases is reported to be enhanced following SAH (52, 54). Furthermore, apocynin attenuated cerebral vasospasm (52, 54), suggesting that NADPH-oxidase-derived ROS may directly contribute to the pathogenesis of cerebral vasospasm following SAH. In addition, evidence suggests that augmented expression of one or more of the NADPH-oxidase subunits may account for the elevation in NADPH-oxidase activity associated with SAH. Indeed, augmented NADPH-oxidase activity was associated with increased expression of $p47^{phox}$, but not Nox2 or $p22^{phox}$, in the membrane fraction of rat cerebral arteries 24 hours after SAH (double hemorrhage), with levels returning to normal by 48 hours (52). The authors of this study concluded that the augmentation in NADPH-oxidase activity was likely due to increased membrane translocation of $p47^{phox}$, and hence assembly and activation of NADPH-oxidase, rather than altered expression of membrane subunits (52). Consistent with this hypothesis, treatment of rats with apocynin decreased membrane translocation of $p47^{phox}$ and superoxide levels following SAH (induced by endovascular perforation of the anterior cerebral artery) (54). In contrast, increased mRNA and protein expression of Nox2 was reported in rat cerebral arteries following SAH (single-injection model), and this was associated with an increase in membrane translocation of Rac (53). Taken together, these studies suggest that augmented NADPH-oxidase activity contributes to the development of oxidative stress and vasospasm during SAH. However, more work is needed to elucidate the precise molecular mechanisms that lead to an increase in NADPH-oxidase activity following SAH.

4.1.5. Stroke

Recent studies have demonstrated a role for NADPH-oxidases in neuronal damage following cerebral ischaemia and reperfusion. For example, Nox2-deficient mice have smaller cerebral infarcts following MCAO than wild-type mice (81, 82). In addition, there is some evidence that NADPH-oxidases may contribute to ROS production and endothelial dysfunction associated with ischaemia and reperfusion. Indeed, rat cerebral artery superoxide production by NADPH-oxidases was ~8-fold greater following stroke (64). Moreover, apocynin improves NO-dependent relaxant responses of rat basilar arteries following hypoxia/reoxygenation *in vitro* (62).

4.2. Cyclooxygenases (COXs), mitochondria and PARP1

COXs have been shown to be an important source of superoxide in the brain and cerebral circulation (14, 83, 84). A very recent study provided both genetic and pharmacological evidence for an important role of cyclooxygenase in the deleterious effects of Ang II. This study showed that Ang II-induced endothelial dysfunction was completely attenuated in prostaglandin E_2 EP1 receptor (EP1R)-deficient mice, and endothelial dysfunction and increases in ROS by Ang II were prevented by an EP1 receptor antagonist or a COX-1 inhibitor. Moreover, these effects were reversed by PGE_2. A COX-2 inhibitor was without effect, suggesting that PGE_2 derived from COX-1 and acting on the EP1 receptor plays a role in mediating oxidative stress and cerebral endothelial dysfunction following Ang II treatment (42). Since Ang II causes oxidative stress and endothelial dysfunction through Nox2-containing NADPH oxidase (see section 4: Sources of Oxidative Stress and Cerebral Endothelial Dysfunction), the authors suggest that constitutive activation of the EP1 receptor by PGE_2 may facilitate Ang II-induced increased in ROS from Nox2-containing NADPH oxidase (42). Such an effect may occur as a consequence of an EP1 receptor-mediated increase in intracellular Ca^{2+} which is needed for NADPH-oxidase activity (42). The mitochondria may be a particularly important source of superoxide in the cerebral vasculature, because the mitochondrial content in cerebral endothelium is relatively high (85). Indeed, endothelial dysfunction of the basilar artery was enhanced in Ang II-

treated MnSOD-deficient when compared with the effect of Ang II in wild-type mice, suggesting that MnSOD normally protects the vasculature during disease states in which Ang II contributes to cerebral vascular dysfunction (47). Ang II is an important mediator of disease in hypertension, but also in diabetes, atherosclerosis and aging, and can stimulate ROS production from mitochondria (86). Not only is mitochondrial oxidative stress important in response to Ang II, several studies, including in humans, suggest that mitochondrial dysfunction, increased oxidative stress and neuronal death are prominent in Alzheimer's disease (reviewed in (87)). Reactive oxygen species signaling in mitochondria also occurs in ischemic brain injury (reviewed in (88)), and is demonstrated by findings such as increased infarct volume and neuronal apoptosis following cerebral ischemia in mice deficient in MnSOD (89). This is also important in periods of ischemia that occur following SAH, where mitochondria can excessively produce free radicals (90). Furthermore, although to our knowledge this is yet to be addressed in the cerebral circulation, oxidative stress and mitochondrial dysfunction in the vasculature may lead to endothelial dysfunction observed in diabetes (90). Another potential mediator of vascular dysfunction is PARP, a downstream target of ROS (91). Increased levels of superoxide in brain and endothelial dysfunction of pial arterioles in streptozotocin-induced diabetic rats could be reversed by the PARP inhibitor PJ-34 (23). Furthermore, PJ-34 also partially restored severe endothelial dysfunction observed in aged mice (92), suggesting that activation of PARP may contribute to oxidative stress and cerebral endothelial dysfunction in disease states such as diabetes and aging.

4.3. SOD deficiency

Mice deficient in genes encoding protein for SOD isoforms are useful models to study the impact of oxidative stress in various subcellular compartments on endothelial function. In mice lacking cytosolic SOD (CuZnSOD), endothelium-dependent relaxation of the isolated basilar artery was significantly impaired compared to littermate controls (93), whereas basilar artery endothelial function was not impaired in mice deficient in mitochondrial SOD (MnSOD) (47, 92, 94). In cerebral arterioles, deficiency in MnSOD resulted in impaired endothelium-dependent responses versus controls (94), although ECSOD deficiency was without effect (46). These studies suggest that different subcellular compartments may contribute to oxidative stress and endothelial dysfunction in different blood vessels of the cerebral circulation.

4.4. DDAH Deficiency

As mentioned, endothelial levels of NO are in large part due to synthesis by eNOS, which produces NO and L-citrulline using L-arginine as a substrate. NO synthesis can be selectively inhibited by guanidine-substituted analogues of arginine, including the endogenous inhibitor of eNOS, asymmetric dimethylarginine (ADMA). Dimethylarginine dimethylamine hydrolase (DDAH) hydrolyses ADMA into L-citrulline and dimethylamine (95). ADMA is considered an independent marker for acute stroke and transient ischemic attacks (96) and has been reported to inhibit endothelium-dependent cerebral vasodilation (97, 98) and cerebral perfusion in humans (99). In humans, 80% of ADMA generated is metabolised by DDAH (100). Thus DDAH, which is expressed in vascular tissue (101-103) represents a potentially important pathway preserving NO bioavailability. Furthermore, DDAH is an oxidant-sensitive enzyme, and DDAH activity is reduced during oxidative stress (103, 104). A recent study reported that cerebral endothelial dysfunction by ADMA is prevented in mice overexpressing DDAH-1 (98).

5. CONCLUDING REMARKS AND PERSPECTIVE

Over the last decade there have been significant advances in the understanding of mechanisms involved in oxidative stress and endothelial dysfunction in the cerebral circulation in several disease states (Table 1). Experimental evidence indicates NADPH-oxidase may be a major source of pathological ROS, although more work is needed to clarify the relative importance of other potential sources, and the interaction between these ROS sources. This latter point notwithstanding, it is apparent that the identification and development of therapeutic agents that specifically target vascular NADPH-oxidases could represent a novel strategy for the treatment and prevention of diseases affecting the cerebral circulation.

6. ACKNOWLEDGEMENTS

The authors are grateful for the following funding support from the National Health and Medical Research Council of Australia (NHMRC): Dr Sophocles Chrissobolis is supported by a CJ Martin Fellowship; Drs Alyson Miller and Grant Drummond are supported by Career Development Awards and Project Grants; Dr Barbara Kemp-Harper is supported by Project Grants; and A/Prof Chris Sobey is a NHMRC Senior Research Fellow and is supported by Project Grants.

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Abbreviations: EDR (endothelium-dependent relaxation); ROS (reactive oxygen species); R^+/A^+ (mice expressing human renin and angiotensinogen); MnTBAP (Mn(III) tetrakis (4-benzoic acid) porphyrin chloride); PEG-SOD (polyethylene glycol superoxide dismutase); STZ (streptozotocin); OLETF (Otsuka Long-Evans Tokushima Fatty); SAH (subarachnoid hemorrhage); ACA (anterior communicating artery); PPARγ (peroxisome proliferator-activated receptor gamma); APP (amyloid precursor protein); CuZnSOD (copper-zinc superoxide dismutase); MnSOD (manganese superoxide dismutase); ECSOD (extracellular superoxide dismutase); SHRSP (stroke-prone spontaneously hypertensive rat); MCAO (middle cerebral artery occlusion).

Key Words: Cerebral Vascular, Oxidative Stress, Endothelial Dysfunction, Hypertension, Stroke, Alzheimer's Disease, Diabetes, Subarachnoid Hemorrhage, Angiotensin II, NADPH oxidase, Review

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