LEADING ARTICLE

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Reactive Oxygen Species in the Cerebral Circulation

Physiological Roles and Therapeutic Implications for Hypertension and Stroke

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Abstract

It is now clear that reactive oxygen species (ROS) can act as signalling molecules in the cerebral circulation under both physiological and pathological conditions. Some major products of superoxide ($O_2^{\bullet-}$) metabolism, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), appear to be particularly good cerebral vasodilators and may, surprisingly, represent important molecules for increasing local cerebral blood flow.

A major determinant of overall ROS levels in the cerebral circulation is the rate of generation of the parent molecule, $O_2^{\bullet-}$. Although the major enzymatic source of $O_2^{\bullet-}$ in cerebral arteries is yet to be conclusively established, the two most likely candidates are cyclo-oxygenase and nicotinamide adenine dinucleotide phosphate (reduced form) [NADPH] oxidase. The activity of endogenous superoxide dismutases (SODs) play a vital role in determining levels and effects of all individual ROS derived from metabolism of $O_2^{\bullet-}$.

The term 'oxidative stress' may be an over-simplification that hides the complexity and diversity of the ROS family in cerebrovascular health and disease. Although a generalised increase in ROS levels seems to occur during several vascular disease states, the consequences of this for cerebrovascular function are still unclear.

Because enhanced breakdown of $O_2^{\bullet-}$ by SOD will increase the generation of the powerful cerebral vasodilator H_2O_2, this latter molecule could conceivably act as a compensatory vasodilator mechanism in the cerebral circulation under conditions of elevated $O_2^{\bullet-}$ production.

Some recent clinical data support the concept of a protective role for cerebrovascular NADPH oxidase activity. Although it is quite speculative at present, if NADPH oxidase were to emerge as a major source of beneficial vasodilator ROS in the cerebral circulation, this may represent a significant dilemma for treatment of ischaemic cerebrovascular conditions, as excessive NADPH oxidase activity is associated with the progression of several systemic vascular disease states, including hypertension and atherosclerosis.

Despite data suggesting that antioxidant vitamins can have beneficial effects on vascular function and that their plasma levels are inversely correlated with risk

of cardiovascular disease and stroke, the results of several recent large-scale clinical trials of antioxidant supplementation have been disappointing.

Future work must establish whether or not increased ROS generation is necessarily detrimental to cerebral vascular function, as has been generally assumed, or whether localised increases in ROS in the vicinity of the arterial wall could be beneficial in disease states for the maintenance of cerebral blood flow.

Cerebral blood vessels have a number of unique properties, reflecting the vital importance of maintaining an adequate and relatively constant supply of blood to the brain under a wide range of conditions. One such property is that cerebral arteries demonstrate a different profile of vasomotor responses to reactive oxygen species (ROS) compared with many systemic arteries, and that these ROS appear to often act as important signalling molecules in normal cerebral vasomotor responses.

It is well known that the superoxide anion ($O_2^{\bullet-}$) is an endogenous ROS molecule that reacts avidly with, and inactivates, nitric oxide (NO). This results in the formation of another harmful species, peroxynitrite ($ONOO^-$) [figure 1 and figure 2]. Thus, although $O_2^{\bullet-}$ can potentially dilate cerebral arteries,[1] it is generally assumed that in both systemic and cerebral arteries inactivation of endothelium-derived NO is the predominant vascular effect of $O_2^{\bullet-}$. Hence, because of this powerful action, $O_2^{\bullet-}$ is regarded primarily as a damaging, pro-vasoconstrictor (or anti-vasodilator) ROS molecule.

In contrast, as is described in this article, some major products of $O_2^{\bullet-}$ metabolism, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), appear to be particularly good cerebral vasodilators and they may mediate some physiological dilator responses in the cerebral circulation. Hence, when generated transiently from $O_2^{\bullet-}$ in sufficiently high concentrations, H_2O_2 and $OH^{\bullet}$ may surprisingly represent beneficial ROS molecules for maintaining local cerebral blood flow.

This review first describes evidence for ROS (especially H_2O_2 and $OH^{\bullet}$) as important endogenous cerebral vasodilators, and then briefly outlines current knowledge of alterations that occur in ROS-related cerebral vascular mechanisms in some disease states predisposing to stroke, and some clinical implications and relevant data from clinical trials.

Previous studies of the effects of ROS on cerebral blood vessels fall into two broad categories: (i) those that have directly investigated vasoactive responses to ROS of exogenous origin (e.g. ROS generated via chemical or enzymatic reactions, or prepared in solution); and (ii) those that have studied the role of endogenous ROS in mediating vasoactive effects of other stimuli (e.g. using pharmacological inhibitors of ROS generation or effects; or ROS-related transgenic animals). We begin by briefly describing some findings from both approaches that have enabled increased understanding of the effects of ROS on cerebral artery tone.

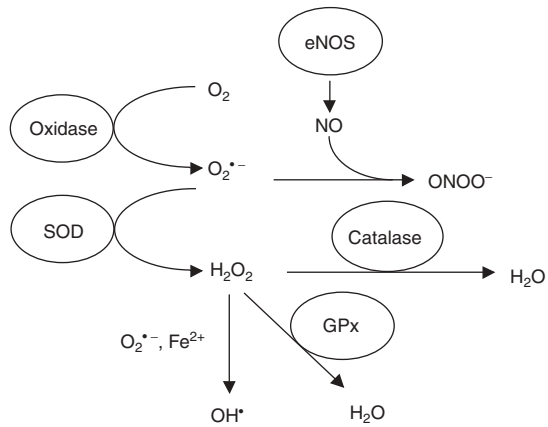

Fig. 1. Generation and breakdown of reactive oxygen species in the vascular wall. The formation of superoxide ($O_2^{\bullet-}$) from molecular oxygen (O_2) is catalysed by vascular oxidases. $O_2^{\bullet-}$ can react rapidly with nitric oxide (NO) produced by endothelial NO synthase (eNOS) to form peroxynitrite ($ONOO^-$). Alternatively, $O_2^{\bullet-}$ can be broken down by various endogenous isoforms of superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). H_2O_2 can in turn be metabolised to H_2O by other endogenous antioxidant enzymes, such as catalase or glutathione peroxidase (GPx). H_2O_2 can also undergo non-enzymatic breakdown in the presence of Fe^{2+}/Fe^{3+} to form hydroxyl radicals ($OH^{\bullet}$) via Haber-Weiss or Fenton chemistry.

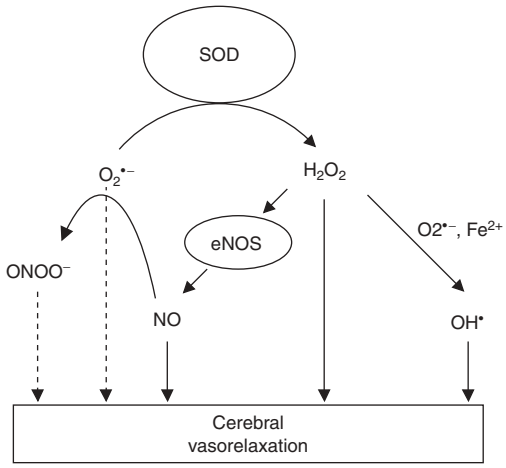

Fig. 2. Potential reactive oxygen species (ROS) stimuli for cerebral vasorelaxation. Superoxide ($O_2^{\bullet-}$), itself reported to have some cerebral vasodilator actions, represents the parent ROS for a number of individual ROS molecules known to possess marked vasodilator effects. Activity of superoxide dismutase (SOD) is thus centrally important for generating increased vascular levels of $O_2^{\bullet-}$ metabolites, including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and, perhaps to a lesser extent, peroxynitrite ($ONOO^-$). In addition, H_2O_2 is a potent stimulus of endothelial nitric oxide synthase (eNOS) gene expression.

1. Effects of Exogenous Reactive Oxygen Species (ROS)

1.1 Enzymatically Generated ROS

Many early investigations into the effects of ROS on vasoactive tone employed exogenous generation of ROS, particularly via the reaction between xanthine and xanthine oxidase which produces $O_2^{\bullet-}$ and H_2O_2. In pial arterioles of anaesthetised cats, application of xanthine/xanthine oxidase produces a rapid-onset (<1 minute) dilatation that is reversible if washed out after 2–4 minutes. The vasodilator response was reduced by the application of either superoxide dismutase (SOD) or catalase, while combined treatment with both scavengers largely prevented the vasodilatation.[2] This result was interpreted as possibly indicating that $O_2^{\bullet-}$ and its metabolic product, H_2O_2, can induce vasodilatation in the cat cerebral vascular bed. An additional explanation is that by reducing the availability of either $O_2^{\bullet-}$ or H_2O_2 by Haber-Weiss chemistry, there will also be a reduction in the level of $OH^\bullet$ (figures 1 and 2). If this were the case in cat pial arterioles, $OH^\bullet$ may also represent a major contributor to the vasodilator response to xanthine/xanthine oxidase. Longer treatment (30 minutes) with xanthine/xanthine oxidase in those studies caused a longer-lasting vasodilatation that was still present 1 hour after washout, an effect that was attributed to ROS-mediated damage of vascular smooth muscle and hence loss of vascular tone, rather than activation of a particular signalling cascade.[2] Because the long-lasting loss of tone could also be inhibited by SOD or catalase, these observations suggested that sustained exposure to ROS damages cerebral vessels *in vivo*.[2] Similarly, in studies of pial arterioles of anaesthetised mice in which acetylaldehyde and xanthine oxidase were used as the ROS-generating system, vasodilatation could be inhibited by scavengers of $O_2^{\bullet-}$, H_2O_2 or $OH^\bullet$, again suggesting that $OH^\bullet$ was the ultimate mediator of relaxation.[1]

1.2 Hydrogen Peroxide

Exogenously applied H_2O_2 exerts powerful effects on cerebral vascular tone. Studies in a number of different species have demonstrated a cerebral vasodilator effect of exogenous H_2O_2 both *in vitro* and *in vivo*. In pial arterioles of newborn piglets, topical application of H_2O_2 causes a biphasic response consisting of an initial vasoconstriction within the first minute of treatment followed by a progressive vasodilatation over the next 20 minutes.[3] These opposing responses appear to occur via different mechanisms, as treatment with cyclo-oxygenase (COX) inhibitors or a prostaglandin H_2/thromboxane A_2-receptor antagonist abolished the vasoconstriction, while the vasodilatation remained unaffected. Neither the constrictor nor dilator responses to direct H_2O_2 application involved the formation of $OH^\bullet$, because treatment with either an iron chelator or additional Fe^{2+} or Fe^{3+} had no effect on the responses.[3] Although the concentration of H_2O_2 used in that study (10 mmol/L) was high, the vasodilatations were reversible upon removal of H_2O_2. Combined with the observation that H_2O_2 did not alter vessel responsiveness to hypercapnia, this led the investigators to conclude that H_2O_2 was not

causing permanent damage to the blood vessel.[3] In anaesthetised cats, much lower concentrations of H_2O_2 (up to 1 μmol/L) were found to elicit concentration-dependent dilatation of cerebral arterioles that was prevented by treatment with the iron chelator deferoxamine, implicating $OH^{\bullet}$ as a mediator, whereas dilatation to H_2O_2 (3 μmol/L) was unaffected by deferoxamine.[4] Iida and Katusic[5] found evidence that in dog isolated middle cerebral arteries H_2O_2 (1–100 μmol/L) causes relaxations in part by activation of arachidonic acid metabolism via the COX pathway with a subsequent increase in cyclic adenosine monophosphate levels and activation of K^+ channels in vascular smooth muscle.[5] Similarly, topical application of H_2O_2 (1–100 μmol/L) is reported to cause concentration-dependent dilatation of rat[6,7] or rabbit[8] pial arterioles *in vivo* via opening of either large conductance calcium-activated K^+ channels, or adenosine triphosphate (ATP)-sensitive K^+ channels, respectively. Thus, the mechanisms mediating cerebral vascular responses to H_2O_2 may differ depending on the animal species studied, the duration of exposure, and the local concentrations of H_2O_2 that can be achieved under endogenous conditions.[4]

1.3 Peroxynitrite

In one interesting study, Wei et al.[8] have reported that, like $O_2^{\bullet-}$ and H_2O_2, topical application of $ONOO^-$ can also induce dilatation of rabbit cerebral arterioles *in vivo*. In that study $ONOO^-$, which decomposes rapidly in aqueous solution, was prepared as a stable solution at pH 14, and then within 3 seconds was added to an appropriately buffered solution of mock cerebrospinal fluid and applied to the brain surface at a final pH of 7.3. The vasodilator responses observed were found to be sensitive to the ATP-sensitive K^+ channel inhibitor glibenclamide (glyburide).[8] The mechanism by which $ONOO^-$ opens ATP-sensitive K^+ channels was not investigated, and it is possible that a breakdown product of $ONOO^-$ (such as $OH^{\bullet}$) could be responsible for channel opening. Alternatively, $ONOO^-$ is known to react with chemical species such as thiols and uric acid to form products which may act as NO donors, thus implicating NO as a potential mediator for this response.[9,10]

2. ROS as Endogenous Cerebral Vasodilators

Efforts to reach general conclusions about the overall actions of ROS in the cerebral vasculature are complicated by both the complex, dynamic and transient nature of individual ROS signalling, as well as the differing experimental methodologies used in studies of ROS. Nevertheless, it is now clear that ROS can act as signalling molecules in the cerebral circulation under both physiological and pathological conditions.

Although the topical application of exogenous ROS to cerebral arteries provides a convenient means to initially assess the direct functional effects of ROS in the cerebral circulation, such studies can provide only limited information on the physiological roles of ROS as mediators of cellular signalling. The first direct evidence that ROS may act as endogenous (and physiological) signalling molecules in the cerebral circulation came from a study by Kontos et al.,[11] which investigated the responses of cat cerebral arterioles to topical application of the vasodilators sodium arachidonate and bradykinin. These investigators found that the combination of SOD and catalase inhibited vasodilatation caused by either agent, indicating dependence on the generation of $O_2^{\bullet-}$ and/or H_2O_2 (or a derivative species such as $OH^{\bullet}$).[11] Such a role for ROS in mediating responses to both arachidonate and bradykinin has now been confirmed in cerebral arteries of a number of species, including cat, rat and mouse.[6,7,11,12] Consistent with the initial observations in the cat, treatment of rabbit cerebral arterioles with SOD and catalase also reduces arachidonic acid-induced vasodilatation.[13] Sodium arachidonate-induced dilatation of cerebral arterioles in anaesthetised rats is similarly abolished by catalase, and partly inhibited by the iron chelator deferoxamine, indicating roles of H_2O_2 and $OH^{\bullet}$, respectively.[6] In mouse cerebral arterioles, $OH^{\bullet}$ is reportedly the key mediator of bradykinin-induced dilatation, as the responses are blocked by treatment with either deferoxamine or

SOD plus catalase.[12] In contrast, responses to bradykinin in rat pial arterioles appear to be primarily due to H_2O_2, as these dilatations, like those to sodium arachidonate, are abolished by catalase and yet are unaffected by SOD or deferoxamine.[7,14]

It is now clear that the actions of H_2O_2 as a vasodilator are not limited to cerebral blood vessels, with recent studies suggesting that H_2O_2 may be acting as an endothelium-derived hyperpolarising factor in some peripheral vascular beds, including the mesenteric arteries of mice[15] and humans,[16] and the coronary vasculature of dogs[17] and pigs.[18] Importantly, a copper-containing isoform of SOD (SOD1) was shown to play a pivotal role in the endothelial production of H_2O_2.[19]

3. Regulation of ROS Levels in the Vascular Wall

Clearly, if different ROS can potentially exert different kinds of actions on vascular tone in the cerebral circulation, determinants of the balance between levels of individual ROS within the vessel wall will play a critical role in the net vasomotor response. For example, the balance between $O_2^{\bullet-}$ and H_2O_2 will be determined by three major factors: (i) the production rate of $O_2^{\bullet-}$ by vascular oxidases; (ii) the rate of conversion from $O_2^{\bullet-}$ to H_2O_2 by SOD; and (iii) the removal rate of H_2O_2 by either the actions of enzymes such as catalase and glutathione peroxidase, or from the involvement of H_2O_2 in Haber-Weiss and Fenton chemistry to form $OH^{\bullet}$. The kinetics of specific mechanisms for ROS production and breakdown will thus have important consequences for cerebrovascular tone.

3.1 Enzymatic Sources of ROS

A major determinant of overall ROS levels in the cerebral circulation will obviously be the rate of generation of the parent molecule, $O_2^{\bullet-}$. Although the major enzymatic source of $O_2^{\bullet-}$ in cerebral arteries is yet to be conclusively established, the two most likely candidates are COX and nicotinamide adenine dinucleotide phosphate (reduced form) [NADPH] oxidase.

3.2 Cyclo-oxygenase

Interestingly, indomethacin (indometacin) commonly inhibits the catalase-sensitive cerebral vasodilator responses to arachidonate and bradykinin described in section 2, suggesting that COX activity is essential for ROS-mediated dilatation after arachidonate treatment.[6,7,13] The proposal that COX activity directly generates ROS is further supported by a recent study which used lucigenin-enhanced chemiluminescence to directly measure $O_2^{\bullet-}$ production following arachidonic acid treatment of the isolated rabbit basilar artery.[20] Arachidonic acid increased $O_2^{\bullet-}$ generation from the basilar artery, and this effect was significantly attenuated by indomethacin.[20] However, although COX is often regarded as a potential source of ROS in the vasculature, there is, to our knowledge, no direct evidence in the literature demonstrating that this enzyme can produce $O_2^{\bullet-}$ in vascular cells. Therefore, it is difficult to definitively ascertain whether this means that COX itself is directly responsible for the production of ROS, or that COX is merely a critical step in a signalling pathway leading to activation of other ROS-producing enzymes. By analogy, lipoxygenase-derived products of arachidonic acid metabolism are reported to cause activation of NADPH oxidase (see section 3.3) to generate $O_2^{\bullet-}$ in non-cerebral vascular smooth muscle cells.[21] Whether prostanoid products of COX activity might similarly elicit ROS production by other enzyme systems remains to be determined. The situation is further complicated by the observation that $ONOO^-$ (produced by the reaction between NO and $O_2^{\bullet-}$) activates COX activity and prostaglandin biosynthesis *in vitro*.[22] Whether this also occurs in the vasculature, and how such a phenomenon would affect other COX-ROS signalling mechanisms, is unknown.

3.3 Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form) Oxidase

NADPH oxidases are a family of membrane-associated, multi-subunit enzyme complexes that catalyse the formation of $O_2^{\bullet-}$. Like the 'classical' NADPH oxidase found in phagocytes,[23] the vascu-

lar enzyme forms $O_2^{\bullet-}$ by transferring electrons from NADPH or nicotinamide adenine dinucleotide (reduced form) [NADH] to molecular oxygen via flavins contained within the enzyme.[21] NADPH oxidases are found throughout the vascular wall, in endothelial cells, vascular smooth muscle cells and adventitial fibroblasts.[21] Furthermore, a number of different isoforms of NADPH oxidase have been identified in the vasculature.[24] These isoforms differ depending on the identity of the membrane-bound subunit responsible for electron transfer. Classically, electron transfer occurs via the flavin-containing subunit gp91phox, however homologues of this subunit (Nox1 and Nox4) have been shown to be present in the active NADPH oxidases found in vascular cells.[25-27] The vascular NADPH oxidase is activated by a variety of hormonal and mechanical stimuli. A range of endogenous agents such as angiotensin II (see section 5), thrombin, platelet-derived growth factor and arachidonic acid have been reported to stimulate $O_2^{\bullet-}$ production by NADPH oxidase, particularly in vascular smooth muscle cells.[21] In cultured endothelial cells, increases in shear stress are able to increase NADPH oxidase activity,[28] although responses to dynamic mechanical stress *in vivo* are yet to be reported. Any future demonstration of an ability of NADPH oxidase activity to be altered by haemodynamic changes (e.g. mechanical stimuli caused by changing intravascular blood flow) would thus provide significant further insight into the physiological regulation of this enzyme.

Recent evidence suggests that NADPH oxidase is present and likely to be a major source of $O_2^{\bullet-}$ in cerebral arteries. Application of excess substrate for this enzyme (NADPH) to the basilar artery *in vitro* causes a marked increase in $O_2^{\bullet-}$ production,[29,30] which is prevented by the flavin antagonist and NADPH oxidase inhibitor, diphenyleneiodonium. Thus, it appears that NADPH oxidase is expressed constitutively in cerebral blood vessels, and that its activity can be enhanced by application of substrate. Moreover, application of NADH or NADPH to rabbit and rat cerebral arteries causes vasodilatation *in vivo*, which can be inhibited by diphenyleneiodonium, diethyldithiocarbamate (to inactivate copper-containing SODs, SOD1 and SOD3), catalase or tetraethylammonium (an inhibitor of calcium-activated K^+ channels).[29,30] Thus, these observations support a functional role for NADPH oxidase-derived ROS, particularly H_2O_2, in vasodilator responses in the cerebral circulation. The exact isoform of NADPH oxidase responsible for this effect in the cerebral circulation is yet to be conclusively determined, although there is evidence to suggest that an isoform containing the Nox4 subunit is involved.[29]

Although it is quite speculative at present, if NADPH oxidase were to emerge as a major source of beneficial vasodilator ROS in the cerebral circulation, this may represent a significant dilemma for treatment of ischaemic cerebrovascular conditions, as excessive NADPH oxidase activity is associated with the progression of several systemic vascular disease states, including hypertension and atherosclerosis (see section 4). Alternatively, if the isoform that is primarily responsible for ROS production is found to be unique to the cerebral circulation, this may provide a potential therapeutic target.

3.4 Alternative Enzymatic Sources of Superoxide

In addition to COX and NADPH oxidase, there are a number of other oxidative enzymes (such as xanthine oxidase and isoforms of nitric oxide synthase [NOS]) that may also be responsible for the generation of $O_2^{\bullet-}$ in the cerebral circulation. Although there have been a number of studies investigating the potential role of xanthine oxidase in cerebral ischaemic injury, few have focused on the vascular effects of xanthine oxidase activity. Xanthine oxidase is known to be present in cerebral vessels, and a study of cultured cerebral endothelial cells demonstrated that anoxia and re-oxygenation produce $O_2^{\bullet-}$ by the xanthine oxidase pathway.[31] Although many early investigations into the actions of ROS in the cerebral circulation utilised exogenous xanthine/xanthine oxidase as a radical-generating system, our understanding of how endogenous xan-

thine oxidase influences the function of cerebral blood vessels is still limited.

All three isoforms of NOS, that is, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS), have been shown to be capable of 'uncoupling' to produce $O_2^{\bullet-}$ under certain conditions.[32] Of particular relevance to the vasculature is the evidence suggesting that eNOS can generate $O_2^{\bullet-}$ during certain vascular disease states, such as diabetes mellitus and hypertension.[33,34] The transition of eNOS from a NO-generating enzyme to a $O_2^{\bullet-}$-generating enzyme appears to occur when the bioavailability of the NOS co-factor tetrahydrobiopterin is reduced.[35] However, although some studies have demonstrated that supplementation of tetrahydrobiopterin levels can reduce aortic $O_2^{\bullet-}$ production in vascular disease models,[34,36] it is unclear whether a similar phenomenon is present in cerebral vessels.

3.5 Endogenous Superoxide Dismutase

The activity of endogenous SODs presumably plays a vital role in determining levels and effects of all individual ROS derived from metabolism of $O_2^{\bullet-}$. There are three major isoforms of SOD present in blood vessels. The manganese-containing mitochondrial isoform (SOD2) is responsible for only a small proportion of vascular SOD activity, with the copper-containing isoforms SOD1 (cytosolic) and SOD3 (extracellular) accounting for the majority of vascular SOD activity.[37] The relative abundance of various SOD isoforms varies between species and vascular beds, with SOD3 being the predominant isoform in systemic arteries of a number of species, including human and mouse.[37] A recent study in which the isolated rabbit basilar artery was treated with an inhibitor of copper-containing SODs confirmed that the SOD1 and/or SOD3 isoforms are important for limiting $O_2^{\bullet-}$ accumulation in cerebral vessels.[20] Thus, inhibition of copper-containing SOD isoforms in cerebral arteries to increase levels of $O_2^{\bullet-}$ markedly reduced NO-mediated endothelium-dependent vasodilator responses to acetylcholine.[20] Inhibition of copper-containing SODs also attenuated vasodilator responses to bradykinin and arachidonic acid in these vessels, which is consistent with H_2O_2, derived from dismutation of $O_2^{\bullet-}$ by SOD, being a key mediator of the response to these important endogenous factors.[20] Similarly, mice lacking the gene for SOD1 have now been shown to have increased levels of $O_2^{\bullet-}$ and impaired cerebral vasodilatation to authentic NO and also acetylcholine, consistent with a role for endogenous SOD1 in limiting $O_2^{\bullet-}$ concentrations under normal conditions, thus preserving NO and H_2O_2-mediated relaxation.[38] Some studies of cerebrovascular function have shown that application of exogenous SOD can partially restore vasodilator responses to NO-dependent agonists in disease states such as diabetes.[39] A number of other investigators have taken an alternative approach to investigating the role of SOD in the brain, in particular the potential therapeutic use of SOD in the treatment of ischaemic brain injury.[40] Indeed, alteration in the expression and activity of brain SOD (particularly the extracellular isoform, SOD3) may have an impact on local levels of ROS affecting the cerebral circulation. However, regardless of the location, altered levels of SOD will have important implications for the function of cerebral arteries, as enhanced breakdown of $O_2^{\bullet-}$ by SOD will increase the generation of the dismutase product and powerful cerebral vasodilator H_2O_2. H_2O_2 could then conceivably act as a compensatory (i.e. beneficial) 'back-up' vasodilator mechanism in the cerebral circulation under conditions of elevated $O_2^{\bullet-}$ production (see section 4).

4. Hypertension and Cerebral Circulation

Hypertension is a complex and multifactorial disease characterised by both functional and structural changes within the vascular wall. There is now a wide body of evidence that implicates elevated levels of ROS, often referred to as 'oxidative stress', in the pathogenesis of hypertension.[41] Chronic hypertension has profound clinical implications for the cerebral circulation, as this condition is a major risk factor for all subtypes of stroke.[42] Hypertension results in a number of changes to intracerebral vas-

culature, including increased permeability of the blood-brain barrier leading to focal oedema, and endothelial damage that facilitates thrombus formation.[43]

Cerebral vasoconstrictor responses to agents such as serotonin appear to be enhanced in chronic hypertension, a similar abnormality to that which may contribute to the development of cerebral vasospasm after subarachnoid haemorrhage (SAH).[44] Moreover, responses to endothelium-dependent vasodilator agonists such as acetylcholine and bradykinin are impaired in hypertension.[44] Since responses to endothelium-independent stimuli such as sodium nitroprusside are preserved during chronic hypertension, the vascular dysfunction appears to be occurring at the level of the endothelium and not the underlying smooth muscle.[44] Indeed, such endothelial dysfunction is a hallmark of hypertension, and has been observed in cerebral arteries in a variety of models of chronic experimental hypertension, whether genetic, surgical or pharmacologically induced.[44]

A number of studies have attempted to identify the mechanism(s) underlying cerebral artery endothelial dysfunction in vascular diseases such as hypertension. Reduced NO bioavailability due to increased $O_2^{\bullet-}$-mediated NO inactivation is thought to be a major contributor to endothelial dysfunction in the systemic circulation, and may also contribute to this phenomenon in cerebral arteries. However, particularly in the cerebral circulation the degree of potential harm versus benefit of other $O_2^{\bullet-}$-derived vasoactive ROS (e.g. H_2O_2 and $OH^{\bullet}$) perhaps needs special consideration. For example, ROS-mediated vasodilatation could be beneficial by maintaining cerebral blood flow, particularly during cerebral ischaemic episodes common in stroke. As discussed in section 3, the functional consequences of increased ROS levels in the cerebral circulation will depend on the dominant individual ROS.

4.1 Is Cerebrovascular ROS Generation Protective in Hypertension?

Our recent data suggest that NADPH-induced cerebral vasodilatation *in vivo* is enhanced in chronic hypertension, and that this is associated with increased NADPH oxidase activity and expression of the Nox4 catalytic subunit of this vascular enzyme.[29] Furthermore, both diphenyleneiodonium and catalase constrict the basilar artery of hypertensive but not normotensive rats, also consistent with a protective role of NADPH oxidase activity in hypertension.[29] Such findings in the cerebral circulation during chronic hypertension could be consistent with NADPH oxidase-mediated vasodilatation being a compensatory mechanism invoked to help maintain cerebral perfusion even in the setting of high levels of circulating angiotensin II. Whether such findings are applicable in stroke is currently unknown. Mice with the gp91phox gene (a critical component of neutrophil NADPH oxidase) deleted show reduced cerebral infarction volume following middle cerebral artery occlusion when compared with wild-type littermates.[45] Although this is supportive evidence for the detrimental effects of high levels of neutrophil-derived ROS, the role of vascular NADPH oxidases in stroke is currently unknown. Indeed, it is possible that NADPH oxidase-mediated vasodilatation as a result of a continuous low level of ROS production could be beneficial by maintaining cerebral blood flow, particularly during the ischaemic episodes of stroke.

Recent clinical data that also support the concept of a protective role for cerebrovascular NADPH oxidase activity are that the C242T polymorphism of p22phox, a membrane-associated subunit of NADPH oxidase, is a novel risk factor for ischaemic cerebrovascular disease[46] and is independently associated with decreased NADPH oxidase activity.[47] Taken together, these recent experimental and epidemiological data are compatible with an important protective role for NADPH oxidase in the cerebral circulation. Nevertheless, there is some clinical evidence that patients with a history of hypertension have impaired recovery from acute stroke when compared with ischaemic stroke patients without a history of hypertension.[48] However, because of the numerous detrimental vascular changes associated with this disease, any beneficial effect of increased cerebral vasodilatation by higher levels of ROS (i.e.

H_2O_2 and $OH^{\bullet}$) generated as a result of NADPH oxidase activity would probably be difficult to detect. It could be argued that outcomes in hypertensive patients might be even worse if cerebral artery NADPH oxidase activity was not elevated. In future studies it will be important to test more directly whether NADPH oxidase expression is protective in the cerebral circulation during experimental stroke. Moreover, there is a need to investigate the long-term consequences of enhanced ROS generation in the cerebral circulation during hypertension as these are still not well described or understood.

5. Angiotensin II and ROS Generation in Cerebral Arteries

Angiotensin II is an octapeptide that forms the major active component of the renin-angiotensin system. It can exert multiple effects on the vasculature under both physiological and pathological conditions, including vasoconstriction, activation of matrix remodelling and modulation of cell growth.[49] Although there have been many investigations into the role of the renin-angiotensin system in the pathogenesis of hypertension, few studies have investigated the extent to which angiotensin II contributes to altered cerebral artery function and structure. Hence, the direct vasoactive effects of angiotensin II in the cerebral circulation are not well characterised but, unlike in the systemic circulation where it is known to be a powerful vasoconstrictor, studies have variously reported constriction of cat cerebral arterioles[50] and dilatation of rat and rabbit pial arterioles[51,52] in response to short-term application of angiotensin II.

More recently, carotid artery function has been studied in transgenic mice that are hypertensive because of over-expression of both human renin and human angiotensinogen.[53] In vessels from these animals there is selective attenuation of responses to acetylcholine, providing strong evidence that hypertension specifically due to increased activation of the renin-angiotensin system produces endothelial dysfunction in the cerebral vasculature.[53]

Many *in vitro* and *in vivo* studies in non-cerebral vessels have reported that angiotensin II is a powerful stimulus of both activity and expression of NADPH oxidase.[54,55] Therefore, it is possible that a similar mechanism of NADPH oxidase activation by angiotensin II may exist in cerebral arteries. Evidence to support this idea can be found in a recent *in vivo* study of rabbit cerebral arterioles, which showed that topical application of angiotensin II for 2 hours had no effect on baseline arteriole diameter. However, angiotensin II treatment impaired responses to bradykinin,[56] which in cerebral arterioles appears to induce vasodilatation via the production of H_2O_2.[7,20] The angiotensin II-induced impairment in endothelium-dependent dilatation could be prevented by treatment with either the SOD mimetic tiron or the NADPH oxidase inhibitor diphenyleneiodonium.[56] Although this study did not directly measure levels of ROS in cerebral vessels, it raises some intriguing questions about the actions of angiotensin II and ROS in the cerebral vasculature. Of particular interest is the implication that $O_2^{\bullet-}$ production stimulated by angiotensin II can impair vasodilator responses mediated by other ROS (such as H_2O_2). Such interactions further illustrate the complexity of the vascular angiotensin II-ROS system, particularly when considering situations in which the renin-angiotensin system is altered (whether due to endogenous over-activity in chronic hypertension or anti-hypertensive drug treatment). In light of this, it is interesting to consider that one cerebrovascular adverse effect of ACE inhibitors or angiotensin II receptor antagonists may be the reduced generation of vasodilator ROS. Although ACE inhibitor treatments do appear to have some benefit in reducing stroke incidence amongst high-risk populations,[57] there is still controversy as to whether these effects are simply due to the anti-hypertensive actions of these drugs. Furthermore, there is little evidence to link any of these beneficial effects with reduction in levels of ROS (see also sections 6 and 7).

6. ROS and Experimental Cerebrovascular Disease

6.1 Experimental Stroke

The generation of $O_2^{\bullet-}$ has long been implicated in cerebral ischaemia-reperfusion injury.[58,59] For example, intravenous administration of SOD and catalase (conjugated to polyethylene glycol to increase cell permeability) reduces infarct volume after focal cerebral ischaemia in rats.[60] Similarly, less brain oedema and lesion size occur following focal cerebral ischaemia in transgenic mice that overexpress SOD1 (and in which the rate of H_2O_2 generation would be expected to be higher).[61,62] Conversely, animals deficient in SOD1 activity suffer considerably greater infarct volume, brain swelling, neurological deficits and mortality following 1 hour of cerebral ischaemia.[63] The enzymatic sources of ROS formation in cerebral ischaemia have been suggested to include COX, xanthine oxidase, NO synthase and NADPH oxidase.[64] Thus, these observations provide more direct support for the concept that increased levels of $O_2^{\bullet-}$ *per se* are detrimental to the cerebral circulation, and yet is also generally compatible with a novel concept being raised in this review – that H_2O_2 might be at least somewhat beneficial in supporting brain blood flow under ischaemic conditions.

6.2 Subarachnoid Haemorrhage

SAH, a significant cause of stroke, results from the rupture of a cerebral aneurysm, leading to bleeding and clot formation around major cerebral arteries. Delayed cerebral vasospasm and impaired vasodilatation are critical complications occurring after SAH, and appear to involve the actions of $O_2^{\bullet-}$.[65,66] An increase in $O_2^{\bullet-}$ production associated with cerebral vasospasm has been shown following SAH in dogs; however, the mechanism responsible for $O_2^{\bullet-}$ production was not identified.[67] More recently, results from studies of SAH in rats reported that increased accumulation of NADPH oxidase-derived $O_2^{\bullet-}$ preceded the development of cerebral vasospasm.[68] Furthermore, intracisternal administration of the NADPH oxidase inhibitor, diphenyleneiodonium, prior to the induction of SAH partially inhibited development of cerebral vasospasm.[68] Consistent with these reports, the development of vasospasm following experimental SAH in rabbits can be attenuated by gene transfer of SOD3 into the cisterna magna.[69]

7. Anti-ROS Therapy in Clinical Hypertension and Stroke

Stroke is the third-leading cause of death in the Western world, and is responsible for 10% of all deaths worldwide.[64] Hypertension is the major, and also the most preventable, risk factor for clinical stroke. Although many therapies are being developed in an attempt to reduce neurological injury following stroke, prevention remains the strategy most likely to have the greatest impact in reducing the burden of this disease. However, direct clinical data regarding the potential role of ROS in stroke is limited. A multicentre Japanese trial examining the effects of ebselen (a cell-permeable antioxidant capable of reducing levels of both H_2O_2 and $ONOO^-$) reported that patients who received ebselen achieved a better outcome at 1 month after acute ischaemic stroke than patients who received the placebo.[70] However, the improvement was only significant in patients who started treatment within 24 hours of stroke onset, while at 3 months following stroke there was no difference between the ebselen- and placebo-treated groups, a trend which probably reflects the spontaneous improvement over this time period.[70]

7.1 Vitamin Studies

Thus far, most of the attempts to explore the clinical therapeutic potential of targeting ROS in cardiovascular disease have come from studies of effects of antioxidant vitamins on the pathogenesis of hypertension and the incidence of stroke. For example, in male smokers the risk of intracerebral haemorrhage and cerebral infarction was decreased with high serum levels of vitamin E, while high serum levels of β-carotene decreased risk of cerebral infarction.[42]

Patients experiencing an acute ischaemic stroke have been reported to have decreased plasma antioxidant levels immediately after a cerebrovascular incident. In particular, vitamin C levels are reported to be consistently lower in patients with the worst outcome.[71] Overall plasma antioxidant activity is also associated with high lesion volume and neurological impairment in patients with cerebral infarction.[72] However, these studies provided no information about antioxidant levels prior to acute stroke, so it is difficult to determine if the low antioxidant levels are a cause or consequence of stroke.

A number of studies have investigated associations between vitamin C and the incidence of stroke in various populations. In a 20-year follow-up study, an inverse relationship was found between vitamin C status (whether assessed by dietary intake or plasma level) and mortality from stroke, even after adjustment for differences in diastolic blood pressures and serum cholesterol levels.[73] A prospective cohort study over a 20-year period similarly found that risk factors for all strokes were inversely correlated with serum vitamin C level.[74] The investigators speculated that the beneficial effects of vitamin C were partly mediated by a lowering of blood pressure, as serum vitamin C levels were inversely correlated with baseline blood pressures in this study population.[74] However, a direct effect of vitamin C on blood pressure in humans is yet to be conclusively demonstrated, as a number of randomised trials have produced conflicting results.[75] Nevertheless, an interaction between vitamin C and blood pressure has been further implicated in a recent study which showed that low plasma vitamin C levels increased risk of stroke in hypertensive and overweight men.[76] Thus, the relationships between vitamin C, blood pressure and stroke risk are yet to be fully elucidated, as are the mechanisms underlying the actions of vitamin C.

7.2 Antioxidants in Clinical Trials

Despite the data suggesting that antioxidant vitamins can have beneficial effects on vascular function and that their plasma levels are inversely correlated with risk of cardiovascular disease and stroke, the results of several recent large-scale clinical trials of antioxidant supplementation have been disappointing. The Heart Protection Study recently examined the effects of antioxidant vitamin supplementation on vascular events in 20,500 high-risk individuals.[77] Although the treatment regimen (tocopherol [vitamin E] 600 mg/day, ascorbic acid [vitamin C] 250 mg/day and β-carotene 20 mg/day) increased the concentration of antioxidants in the blood, there was no difference in the incidence of either fatal or non-fatal strokes (or other major vascular events) between patients allocated vitamin or placebo.[77] Likewise, the HOPE (Heart Outcomes Prevention Evaluation) study of 9500 patients at high risk for cardiovascular events found no reduction in deaths from cardiovascular causes, myocardial infarctions or strokes in patients who received tocopherol 400 IU/day for a mean of 4.5 years.[78] The lack of beneficial effects in these large-scale clinical trials, despite promising pre-clinical work, seems to reflect the complexity of interactions between ROS and vascular signalling systems, and perhaps the potential mixture of physiological and pathological actions of ROS. It is also quite possible that the local levels of antioxidant vitamins achieved in the vascular wall clinically are still not high enough to match the excess $O_2^{\bullet -}$ formation in vascular diseases such as stroke. Also worth considering is that some of the beneficial effects of antioxidants (e.g. on vasodilator H_2O_2 levels) may be partially offset by their paradoxical pro-oxidant effects. This is because after scavenging a radical, antioxidants themselves become radical species (e.g. tocopherol → tocopheroxyl radical; ascorbic acid → ascorbyl radical), which may cause lipid peroxidation and depletion of glutathione levels.[79]

7.3 ACE Inhibitors and Angiotensin II Receptor Antagonists in Clinical Trials

Interestingly, and of relevance to the potential involvement of angiotensin II in vascular ROS production (see section 5), despite the lack of beneficial effects of tocopherol on cardiovascular outcomes, the HOPE trial was terminated early because of a clear beneficial effect of the ACE inhibitor, ramipril,

which reduced rates of stroke, myocardial infarction and death due to cardiovascular events.[80] A more detailed analysis of the impact of ramipril on stroke subtypes and outcomes revealed that patients receiving ramipril had not only lower incidence of stroke, but also less functional impairment following non-fatal stroke.[57] Although the epidemiological benefits of blood pressure lowering on stroke risk are seen across a wide range of populations and are generally well accepted,[81] a key finding of the ramipril study was that the benefits were seen over a range of systolic and diastolic blood pressures, and were not limited to patients with hypertension.[57] However, whether the beneficial effects of these medications extend beyond blood pressure lowering remains controversial, as the publication of these studies prompted considerable correspondence in the latter half of 2002. However, further support for the beneficial effects of ACE inhibitors/angiotensin II receptor antagonists came from the recent LIFE (Losartan Intervention For Endpoint reduction in hypertension) study[82] in which patients with essential hypertension and signs of left ventricular hypertrophy were treated with an angiotensin II type 1 receptor antagonist (losartan). A substantial (approximately 25%) decrease in the incidence of fatal and non-fatal strokes was found in losartan-treated patients compared with patients who received atenolol (a β-adrenoceptor antagonist) to lower blood pressure. This again suggests that reducing the activity of the renin-angiotensin system may have beneficial effects beyond the lowering of blood pressure.[82]

7.4 Possible Relevance of Site of ROS Generation

These recent clinical data would not necessarily support the concept raised earlier that inhibiting angiotensin II-induced ROS production in cerebral arteries could be detrimental, even if it is removing a protective compensatory mechanism to support perfusion, perhaps because of the substantial overall systemic benefits achieved by blocking angiotensin II generation by ACE. Perhaps a final thought that is pertinent to any such speculation of the beneficial or detrimental actions of ROS in the brain is that, as cerebral vasodilators, ROS may be beneficial when generated over the short-term in low levels in the vicinity of the cerebral vascular wall and yet, as powerful oxidants, they may be quite toxic to the brain when generated in high levels either for short-term or long-term in or around neuronal cells. This issue could perhaps only be resolved using future experimental approaches to selectively manipulate vascular and/or non-vascular ROS levels in the brain.

8. Conclusion

The actions of ROS in the cerebral circulation and in neuronal tissue appear to have varied and extensive biological significance under normal and pathological circumstances, making it potentially very complicated to predict their clinical relevance. The term ‘oxidative stress’ may be an over-simplification that hides the complexity and diversity of the ROS family in vascular health and disease. As has been discussed throughout this review, individual ROS can exert different (or even opposing) effects in the brain and its blood vessels. The balance between the levels of different ROS molecules will be the main determinant of the effects of ‘oxidative stress’ on vascular function. Such complexities may partially account for the varying but relatively weak effectiveness of general antioxidant treatment strategies thus far tested clinically.

Indeed, there are now data to suggest that a generalised increase in ROS levels occurs during several vascular disease states; however, the consequences of this for cerebrovascular function are still unclear. Is increased ROS generation necessarily detrimental to cerebral vascular function, as has been generally assumed? Or could short-term (or even long-term) low-level localised increases in ROS in the vicinity of the arterial wall be beneficial for the maintenance of cerebral blood flow by the vasodilator actions of H_2O_2 or $OH^{\bullet}$?

It is clear that, despite a recent surge in research, the impact of ROS in the cerebral and non-cerebral vasculature is an area that still has many more questions than answers. Indeed, the majority of our

knowledge about the specifics of ROS signalling in the vasculature comes from animal models, and is yet to be confirmed in a clinical setting. A greater understanding of these actions of ROS, particularly during cerebrovascular disease, should provide new opportunities for targeted prevention and treatment of vascular dysfunction.

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