A new approach for the investigation of reperfusion-related brain injury

C.M. Maier1, L. Hsieh, T. Crandall, P. Narasimhan and P.H. Chan
Department of Neurosurgery, Department of Neurology and Neurological Sciences, and Program in Neurosciences, Stanford University School of Medicine, Stanford University, 1201 Welch Road, MSLS #P357, Stanford, CA 94305-5487, U.S.A.

Abstract
Effective stroke therapies require recanalization of occluded cerebral blood vessels; however, early reperfusion can cause BBB (blood–brain barrier) injury, leading to cerebral oedema and/or devastating brain haemorrhage. These complications of early reperfusion, which result from excess production of ROS (reactive oxygen species), significantly limit the benefits of stroke therapies. Here, we summarize some of the findings that lead to the development of a novel animal model that facilitates identification of specific free radical-associated components of the reperfusion injury process and allows therapeutic interventions to be assessed. In this model, KO (knockout) mice containing 50% activity of the mitochondrial antioxidant manganese-SOD (superoxide dismutase) (SOD2-KO) undergo transient focal ischaemia followed by reperfusion. These animals have delayed (>24 h) BBB breakdown associated with activation of matrix metalloproteinase-9, inflammation and a high brain haemorrhage rate. These adverse consequences are absent from wild-type littersmates, SOD2 overexpressors and minocycline-treated SOD2-KO animals. In addition, using microvessel isolations following in vivo ischaemia/reperfusion, we were able to show that the tight junction membrane protein, occludin, is an early and specific target in ROS-mediated microvascular injury. This new model is ideal for studying ischaemia/reperfusion-induced vascular injury and secondary brain damage and offers a unique opportunity to evaluate free radical-based neurovascular protective strategies.

Introduction
Ischaemic stroke results from the abrupt occlusion of cerebral blood vessels. Early restoration of cerebral blood flow, either by pharmacological thrombolysis or mechanical clot removal, has proven to be the most effective strategy for acute stroke therapy. In 1996, the U.S. FDA (Food and Drug Administration) approved tPA (tissue plasminogen activator), a thrombolytic agent that can dissolve occlusive thrombi, as the first effective stroke therapy. This treatment was tested up to 8 h after symptom onset and successfully retrieved thrombi from within cerebral vessels. The device (Merci Concentric Retriever) that works like a corkscrew to directly retrieve thrombi from within cerebral vessels. The device was tested up to 8 h after symptom onset and successfully retrieved approx. 50% of occluded vessels. Clinical outcomes were significantly improved among patients who had successful recanalization when compared with patients who remained occluded. Although this therapy does not require a thrombolytic agent, it is also associated with a risk of SICH of approx. 10%. The risk of brain haemorrhage associated with both thrombolytic and mechanical clot removal therapies is one of the major limitations of these treatments.

Early reperfusion of ischaemic brain tissue can injure the BBB (blood–brain barrier), leading to cerebral oedema and brain haemorrhage. Brain haemorrhages following reperfusion are particularly devastating and are associated with extremely high rates of morbidity and mortality. In addition to damaging the ischaemic arterial wall and microvasculature, reperfusion can also injure neurons and glial cells [1–3].

Free radicals, specifically ROS (reactive oxygen species) are generated soon after vessel occlusion, as well as in later stages of ischaemic reperfusion. These ROS are the fundamental mediators of reperfusion injury. Cerebral endothelial cells, which make up the BBB, are one of the primary targets. Endothelial cells are abundant with mitochondria and, under physiological conditions, electron transport within mitochondria is responsible for the production of the ROS superoxide anion, which is scavenged by the antioxidant SOD (superoxide dismutase). During reperfusion, both acute and chronic cell injury can result from excessive superoxide anion production [4,5].

Inflammation is another key component of reperfusion injury. When deprived of oxygen, cerebral endothelial cells can release/express various inflammatory mediators, thus initiating an inflammatory response. The vascular endothelium and activated inflammatory cells, such as leucocytes and microglia, are also a source of ROS. When intrinsic cellular...
antioxidant systems become overwhelmed, ROS become activators of cell death mechanisms that cause further injury [6].

Proteinases intensify the cellular damage that follows ischaemic reperfusion. For example, MMPs (matrix metalloproteinases) can degrade extracellular matrix proteins, damaging endothelial cells and increasing BBB permeability [7]. During ischaemia/reperfusion, numerous MMPs and their inhibitors are produced by astrocytes, microglia and endothelial cells. MMP9, also known as gelatinase B, is of particular interest since it is up-regulated in cerebrovascular disorders. Increased MMP9 expression can lead to endothelial cytotoxicity [8], and recent evidence suggests that inhibition of MMP9 results in improved vascular integrity following ischaemia [9,10].

Despite the substantial clinical importance of reperfusion injury, this phenomenon remains poorly understood. One of the reasons for this knowledge gap is that there are very few animal models available to facilitate investigation. In this review, we summarize some of the findings that led to the development of a novel animal model that has great potential to clarify the pathophysiological processes described above as well as evaluate therapeutic strategies aimed at prevention of reperfusion-related brain haemorrhage and oedema (for complete study results, see [11]).

Main findings

ROS overload during ischaemia/reperfusion results in increased susceptibility to brain injury, oedema and haemorrhage and is associated with increased MMP9 levels

Heterozygous SOD2-KO (knockout) mice [12] (CD1/SV129 background, backcrossed for over ten generations), heterozygous SOD2-TG (transgenic) mice [13] (C57BL/6J background, backcrossed for over ten generations) and their respective WT (wild-type) littermates were used for these studies.

We first demonstrated that SOD2-KO mice are more susceptible to ischaemic brain injury, oedema and haemorrhage after ischaemia/reperfusion than their WT littermates. When subjected to 1 h of middle cerebral artery occlusion followed by reperfusion, the mortality rate for the SOD2-KO animals was nearly 90% at the 72 h endpoint due to the occurrence of large intracerebral haemorrhages. In contrast, 72 h mortality rate in the WT animals was only 10%. We then reduced the ischaemia period to 30 min and demonstrated that the SOD2-KO mortality rate was reduced to 30% at 72 h. Histopathology revealed that SOD2-KO animals had a statistically significant increase in infarct size both at 24 and 72 h compared with WT, as well as an increase in the rate of haemorrhagic transformations (SOD2-KO: 8/9; WT: 3/8) at 72 h (by Perl's Iron staining), which was not observed at 24 h. These haemorrhagic transformations, which were mostly absent from WT mice, were generally widespread throughout the ischaemic tissue and often quite large in the SOD2-KO mice, and many were observed in the border zone between infarct and surviving tissue. These results were confirmed by Western-blot analysis, which showed increased brain haemoglobin values, indicating intracerebral haemorrhage, in SOD2-KO compared with WT littermates at 72 h of reperfusion.

Previous work in our laboratory had shown that oxidative stress is involved in mediating BBB disruption during the first 3–7 h of reperfusion after an ischaemic event through MMP activation [14]. In addition, we had demonstrated that SOD2 could reduce apoptotic cell death [15] and attenuate cytochrome c release from the mitochondria to the cytosol, a critical step in the intrinsic mitochondrial-dependent signaling pathway of the cell death programme [16]. Together with the remarkably increased 72 h haemorrhage rate observed in SOD2-KO animals, these findings led us to hypothesize that ischaemia/reperfusion caused increased ROS production in mitochondria, leading to MMP activation, BBB breakdown, vasogenic oedema and delayed ischaemic neuronal damage.

Protection with the anti-inflammatory agent minocycline

To test our hypothesis and validate our model, we treated SOD2-KO mice and their WT littermates with minocycline, a commonly used tetracycline derivative that crosses the BBB and reduces cytochrome c release from mitochondria [17] and has also been shown to attenuate stroke severity [18]. For this study, half the mice (SOD2-KO animals and WT littermates) received minocycline (day 1: at 1 and 4 h post-insult, 45 mg/kg, intraperitoneal; days 2 and 3: single dose of 22.5 mg/kg), while the other half received equal amounts of saline. Minocycline-treated animals (SOD2-KO and WT) had a significant reduction in infarct size at 72 h of reperfusion compared with saline-treated animals. In addition, minocycline treatment reduced the haemorrhage rate (3/11 versus 7/9 saline-treated) and the mortality rate (2/13 versus 4/13 saline-treated) in SOD2-KO as well as MMP9 expression at 24 and 72 h of reperfusion.

Immunohistochemical studies showed that MMP9 immunoreactivity in vascular structures (found throughout the infarcted hemisphere but most prominently in the border zone between infarct and surviving tissue) was significantly stronger in SOD2-KO mice compared with WT littermates. To study further the relationship between ROS production, MMP9 expression and BBB disruption, we injected Evans Blue into the jugular vein of SOD2-KO and WT mice following ischaemia/reperfusion. Extravasation of Evans Blue from vessels, which indicates vascular permeability, was detected throughout the ischaemic hemisphere, and MMP9 immunoreactivity was easily discerned in vessels showing Evans Blue extravasation. MMP9 expression was very prominent in small vessels in the infarct border, but could also be observed in vascular structures within severely damaged areas in the centre of the infarct as well as in larger vessels.
ROS lead to the degradation of endothelial tight junction components

The brain endothelial cells that form the BBB have tight junctions that are critical for maintaining brain homeostasis and restricting permeability. Some of the major components of tight junction complexes are ZO-1 (zonula occludens 1 protein), occludin and claudin-5, all of which are targets of MMP9 degradation (Figures 1A and 1B). Detection of small changes in tight junction components is difficult in whole brain homogenates, so we adapted a microvessel isolation technique that allows us to isolate free radical-mediated BBB-specific events following ischaemia/reperfusion injury. The resulting microvessel pellet was tested for factor VIII (an endothelial cell marker), Neu-N (a neuronal marker), and GFAP (glial fibrillary acidic protein; a glial marker) to ensure a successful isolation.

As expected, we found progressive degradation of ZO-1, occludin and claudin-5 following ischaemia/reperfusion injury. More interesting, however, were the facts that SOD2-KO animals were particularly sensitive to occludin degradation and showed an associated increase in MMP9 expression compared with WT littermates.

To confirm our MMP9 results from the microvessel isolations, we also tested the effects of OGD (oxygen and glucose deprivation) on endothelial cell cultures. Following 8 h of OGD, which results in 50% cell death, and 0–8 h of reoxygenation, endothelial cells from SOD2-KO mice showed a marked increase in MMP9 expression compared with endothelial cell cultures from WT littermates. By 24 h of re-oxygenation, there was a reduction in MMP9 levels in SOD2-KO endothelial cell cultures that was inversely proportional to cell death in those cultures, while WT endothelial cell cultures showed a substantial increase in MMP9 levels and only a small increase in cell death at the same time point.

SOD2 overexpressors are protected against BBB damage following ischaemia/reperfusion

As proof of principle, we subjected SOD2 overexpressors (which have a 2.5-fold increase in SOD2 activity) and their WT littermates to the same ischaemia/reperfusion paradigm and showed that the rate of haemorrhagic transformations at 72 h was quite low in both groups (SOD2-TG: 3/9; WT: 3/9) and due mostly to mechanical damage by the occluding filament. In addition, examination of the microvasculature within the infarcted area showed more TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)-positive cells in microvessels of SOD2-KO mice compared with their WT littermates, whereas finding such TUNEL-positive cells in SOD2 transgenic animals was rare.
Concluding remarks

Early reperfusion can dramatically attenuate brain injury caused by ischaemic stroke. However, restoration of blood flow is often accompanied by reperfusion injury and the risk of intracerebral haemorrhage. Accumulating evidence suggests that ROS are the fundamental mediators of BBB disruption, cerebral oedema and tissue damage following ischaemia/reperfusion. Understanding the mechanisms that modulate the levels of reactive oxygen intermediates and the factors that regulate the enzymes involved in the degradation of BBB components is critical in order to optimally develop pharmacological agents for the treatment/prevention of ischaemic brain damage.

SOD2-KO mice and the newly developed SOD2 over-expressors allow us to examine, in a molecular fashion, the oxidative mechanisms that mediate brain injury following stroke- and mitochondria-related microvascular dysfunctions. In addition, SOD2-KO animals can facilitate the development and evaluation of treatment strategies that diminish the complications of cerebral reperfusion. This is particularly important given the approaches being investigated to improve stroke treatment, including delivering high-flow oxygen to patients prior to vessel recanalization [19]. Oxygen therapy can enhance the formation of ROS and, thus, increase BBB damage, but recent work suggests that this therapy may extend the reperfusion window when treatment is administered during the ischaemic episode [20]. However, the effects of combining oxygen therapy with thrombolytic agents such as tPA or clot retrieval devices have not yet been examined.

Our findings that minocycline treatment led to reduced haemorrhage rates, infarct volumes and mortality demonstrate that pharmacological agents have the potential to attenuate reperfusion-related brain injury and that our model can be used to evaluate putative therapeutic agents. This is an area of growing interest in stroke research and treatment, as evidenced by the recent work on the free radical trapping agent NXY-059 [21,22], which is undergoing worldwide Phase III trials.

The technique for microvessel isolation following in vivo ischaemia/reperfusion in SOD2-KO mice allowed us to verify the existence of microvascular injury and demonstrate that this injury was likely to be mediated by an increase in MMP9 expression (Figure 1C). Furthermore, this technique allowed us to establish that, in endothelial cells, one of the key consequences of mitochondrial oxidative stress and the ensuing overexpression of MMP9 is damage to the tight junction transmembrane protein occludin. Our findings support previous work demonstrating that occludin can be selectively cleaved by metalloproteinases [23] and phosphorylation of occludin appears to be a key regulator of tight junction permeability [24].

Finally, through the availability of SOD2+/- mice, we were able to demonstrate that overexpression of SOD2 has a protective effect on the microvasculature, raising the important question as to whether this increase in mitochondrial antioxidant capacity can enhance cell-survival mechanisms.

This new model of reperfusion-related brain haemorrhage will help us examine the specific molecular and cellular mechanisms that underlie ischaemia/reperfusion-related brain injury. It will also facilitate the evaluation of neurovascular protective strategies as potential adjunct treatments to currently approved stroke therapies and ultimately translate into new therapeutic interventions for stroke patients.

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References


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