Metabolic Reflow as a Therapy for Ischemic Brain Injury

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Abstract Ischemic neuronal damage is a common feature of occlusive strokes, hemorrhagic strokes, and traumatic brain injury. In addition, ischemia can be an anticipated or unanticipated complication of a variety of surgical procedures. Most therapeutic strategies for managing ischemic injury seek to re-establish blood flow, suppress neural metabolism, and/or limit specific cellular injury cascades. An alternative therapeutic approach is to enhance the delivery of metabolic substrates to ischemic tissue. This strategy is typified by efforts to increase tissue oxygenation by elevating the levels of circulating oxygen. Our studies are examining a complementary approach in which the delivery of metabolic substrates is enhanced by facilitating the diffusion of oxygen and glucose from the vasculature into neural tissue during ischemia. This is achieved by increasing the diffusivity of small molecules in aqueous solutions, such as plasma and interstitial fluid. The carotenoid compound, trans-sodium crocetinate (TSC) is capable of increasing oxygen and glucose diffusivity, and our studies demonstrate that TSC increases cerebral tissue oxygenation in the penumbra of a focal ischemic event. In addition, TSC treatment reduces the volume of cerebral infarction in rodent models of both permanent and temporary focal ischemia. This strategy of "metabolic reflow" thus blunts the metabolic challenge in partially-perfused tissue and reduces ischemic neural injury.

Keywords Stroke · Neuroprotection · Oxygen · Diffusion · Metabolic reflow

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Introduction

Ischemic neural damage can occur as a result of most types of stroke and time is of the essence for managing the ischemic insult to affected cells. Consequently, a primary goal for any therapeutic regimen is to reestablish blood flow to the ischemic tissue. In the case of occlusive (i.e. embolic and thrombotic) strokes, disruption of the intravascular impediment to blood flow, typically utilizing tissue plasminogen activator, is the principal therapeutic modality. In the case of hemorrhagic strokes, the situation is more complex because of the intrinsic challenges to stabilizing the hemorrhagic event. Subarachnoid hemorrhages resulting from the rupture of aneurysms on large cerebral arteries can be stabilized by surgical clipping or intravascular coiling. In contrast, intracerebral hemorrhages are less amenable to treatment. The management of hemorrhagic strokes is further complicated by the effects of residual extravascular blood in the subarachnoid and intraparenchymal spaces. Finally, downstream compromise to the microvasculature plays a role in the ischemic challenge produced by both occlusive and hemorrhagic strokes. These smaller vessels, although sometimes not directly impacted by an occlusion or hemorrhage are a key emerging target for optimizing blood supply after stroke.

Differential diagnosis of hemorrhagic versus occlusive stroke is a standard feature of the emergent response to stroke in order to ascertain whether thrombolytic therapy is appropriate to reestablish blood flow. Although this diagnosis consumes crucial time during an ongoing stroke, it is essential to limit the complications of rebleeding and hemorrhagic transformation. Consequently, an important goal for current experimental and clinical studies is to identify therapeutic modalities that can be administered rapidly and safely irrespective of stroke type. The predominant experimental approach to this problem focuses the inhibition of critical injury cascades. Numerous candidate agents targeting a wide variety of injury mechanisms have been

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identified and have shown considerable promise in preclinical studies of stroke. Unfortunately, human studies directed toward the inhibition of specific mechanisms of neural injury have not proven successful in advanced stage clinical trials. The reasons for these failures are several, including inadequate preclinical testing [9, 10, 29, 35, 38, 44, 53], and underpowered or inappropriately designed clinical trials [7, 10, 12, 15, 16, 26, 54]. Another plausible explanation for this lack of efficacy in late stage clinical trials is that drugs targeting a narrow range of injury mechanisms are incapable of overcoming the broad range of injury cascades that are set in motion by prolonged ischemia [60]. The future of neuroprotective therapy in the clinic may thus ultimately require a multimodal, poly-pharmacological approach to achieve success [41].

Two alternative approaches to limiting ischemic cellular injury involve either the suppression of overall metabolic demand [61] or the enhancement of metabolic supply [57]. The former approach seeks to broadly limit mechanisms of cellular injury by reducing metabolic demand and slowing the rates at which injury cascades can progress. The latter approach is also directed toward a broad inhibition of cellular injury, but does so by increasing metabolic supply to ischemic tissue and thus blunting the overall ischemic challenge. The studies described herein will focus on a novel form of the metabolic enhancement strategy using a pharmacologic intervention to increase the delivery of metabolic substrates to ischemic neural tissue.

Historically, therapeutic strategies to enhance the metabolic supply of ischemic tissue have generally utilized increased systemic oxygenation to improve tissue oxygenation. Hyperbaric and normobaric oxygenation have shown promise in limiting cellular injury and neurological function in animals models of stroke [4, 5, 11, 13, 18, 19, 24, 25, 28, 37, 39, 45, 47, 50, 51, 55–57, 59, 62]. In contrast, clinical trials have been more limited and have provided mixed outcomes to date [1–3, 6, 17, 32–34, 43, 48, 49, 63]. These mixed clinical outcomes can be ascribed in part to small sample sizes and relatively long delays prior to initiating treatment.

The underlying concept for the preceding oxygen enhancement studies is that the elevation of vascular oxygen raises the concentration gradient between blood and tissue and thus increases the movement of oxygen into the tissue compartment. However, it is important to note that the rate of movement of a small molecule, such as oxygen or glucose, is also dictated by its diffusion coefficient in the host medium. In the case of circulating blood, the plasma boundary layer is a key resistance for the movement of molecules from the vasculature into tissue [22, 23]. The diffusivity of oxygen and glucose in an aqueous solution, such as plasma, is dictated in part by the number of hydrogen bonds and intermolecular spacing among water molecules. An increase in hydrogen bonding builds the "structure" of aqueous solutions and facilitates the diffusion of small molecules. It is thus possible to increase the access of vascular oxygen and glucose to tissue by modifying the diffusivity of these metabolic substrates. Trans-sodium crocetinate (TSC) is a carotenoid compound that has previously been shown to increase the diffusivity of small molecules, including oxygen and glucose, by facilitating structure building in aqueous media [27, 52]. Moreover, TSC has been shown to increase tissue oxygenation in multiple organs and to improve survival in a model of hemorrhagic shock [36, 42, 46]. The studies described herein examined the ability of TSC to increase cerebral oxygenation in areas of partial ischemia and its effects on neural injury in permanent and temporary models of focal brain ischemia [30].

Materials and Methods

All experimental protocols were approved by the University of Virginia Animal Care and Use Committee. Adult male Sprague-Dawley rats (330–370 g) underwent permanent or temporary focal ischemia [20] by clipping both common carotid arteries and the left middle cerebral artery (three-vessel occlusion: 3-VO). Using the permanent ischemia paradigm, TSC was administered at one of eight dosages, ranging from 0.023 to 4.580 mg/kg ($n = 7$ animals per group). Equivalent volumes of either Vehicle or TSC were injected into the femoral vein using a "bolus-infusion-bolus protocol", as per the protocol of Okonkwo et al. [36]. Using this protocol, a bolus injection of 0.1 ml was administered 10 min after the onset of ischemia, followed by continuous infusion at 0.01 ml/min for 60 min. Thirty minutes after the end of infusion, another 0.1 ml bolus was injected. The dosages described here represent the total dosage of TSC administered using the bolus-infusion-bolus protocol. After 24 h of permanent ischemia, the animals were euthanized under deep anesthesia. The brains were sectioned coronally at a thickness of 2 mm and the sections were stained in 2% 2,3,5 triphenyltetrazolium chloride (TTC) in phosphate-buffered saline for 5 min at 37°C. Infarct size was measured and the total volume of infarction was corrected for swelling [30].

In the temporary model of focal ischemia, the 3-VO was maintained for 2 h after which the vessels were unclipped and reflow was established. These animals were euthanized at 22 h after establishing reflow. TSC was administered, as described above, beginning 10 min after the onset of ischemia. A dosage of 0.092 mg/kg was used based on the results of the dose-response study using the permanent ischemia model.

The effect of TSC on partial tissue oxygen levels (PtO2) was also examined using the same temporary model of focal ischemia. A Licox probe was placed in the penumbra of the focal ischemic area in order to record tissue oxygenation. TSC was administered at a dosage of 0.92 mg/kg using the bolus-infusion-bolus protocol with the first bolus administered 10 min after the onset of ischemia. The levels of PtO2 were recorded prior to ischemia for at least 20 min to obtain a stable, normoxic baseline. All recorded values were then normalized to this pre-ischemic baseline.

Results

Effect of TSC on Cerebral Infarction After Permanent Focal Ischemia

Treatment with TSC produced a dose-dependent reduction in cerebral injury. The dose-response curve was U-shaped with dosages ranging from 0.023 to 0.229 mg/kg producing significant reductions in infarct volume [30]. The most effective dosage, 0.092 mg/kg, reduced infarct volume by 58%.

Effect of TSC on Cerebral Infarction After Temporary Focal Ischemia

The optimal dosage of TSC for producing neuroprotection in the preceding dose-response experiment was tested for its effect on cerebral infarction produced by temporary (2 h) focal ischemia followed by 22 h of reperfusion. TSC treatment at a dosage of 0.092 mg/kg reduced infarct volume by 45% in this model of ischemia-reperfusion [30].

Effect of TSC on Oxygenation in the Ischemic Penumbra

Partial tissue oxygen levels in the ischemic penumbra of the 3-VO model were reduced by an average of 40–45% from baseline when ischemia was initiated [30]. Ten minutes after the onset of ischemia, animals were treated with either TSC or saline. In the TSC-treated animals, tissue oxygenation began to increase within approximately 10 min of the initial bolus injection. By the end of the second hour of ischemia PtO2 in the penumbra of saline-treated animals was reduced by an average of 41% below baseline, while oxygen levels had recovered to only 20% below baseline in the TSC-treated animals [30]. Upon reperfusion, tissue oxygenation increased well above baseline in both Vehicle-treated and TSC-treated animals. However, tissue hyperoxygenation during reperfusion was significantly less pronounced in TSC-treated animals than in Vehicle-treated animals.

Discussion

During an occlusive stroke, the ischemic penumbra is an area of partial blood flow that contains tissue at risk of being damaged and possibly recruited into cerebral infarction. The penumbra is generally viewed as a region that can be salvaged if vascular reperfusion is achieved within an adequate time frame and/or appropriate measures are taken to protect the tissue. The key determinants of how well penumbral tissue will withstand an ischemic challenge are the duration and depth of the ischemic event. Consequently, when it is feasible, a centerpiece of stroke management includes efforts to restore blood flow in order to limit the duration of metabolic challenge. Thrombolytic therapy can produce complete or partial recanalization with delays to reflow ranging from minutes to several hours after treatment [8, 40]. The delay to recanalization is compounded by the delay to receiving treatment, which typically is on the order of a couple of hours. Therefore, it is not unusual for stroke patients to experience ischemic events that persist for several hours. Although not always effective in establishing reflow, thrombolytic therapy remains the principal medical means for recanalization and a valuable strategy for curtailing the duration of an occlusive stroke.

The depth of an ischemic event can vary widely and the intensity of this challenge dictates the involvement and time course of various injury mechanisms [31]. Our current studies are examining a therapeutic approach designed to blunt the impact of partial ischemia by enhancing metabolic supply to at-risk tissue. Thus, this approach functionally attenuates the depth of ischemic challenge to the penumbra. Trans-sodium crocetinate was shown to substantially and significantly enhance tissue oxygenation in the penumbra of ongoing focal ischemia in the brain [30]. Moreover, TSC reduced neural damage in both permanent and temporary models of focal cerebral ischemia [30]. The protective actions of TSC are predicated on the concept of metabolic reflow, in which ischemic damage is attenuated by facilitating the delivery of metabolic substrates to at-risk tissue. The enhancement of tissue oxygenation is thought to result from the ability of TSC to increase the diffusivity of oxygen [52]. Nonetheless, in studies of this type, it is important to consider alternative explanations for the protective actions of any candidate therapy. Relevant to this issue are previous studies

examining the effects of TSC's structurally-similar parent compound, crocetin. These studies showed that crocetin did not exert significant effects on oxyhemoglobin saturation, oxygen solubility in blood, or blood flow [14, 21]. An increase in the diffusivity of metabolic substrates thus remains a parsimonious explanation for the observed increases in tissue oxygen and the resultant neural protection.

As discussed earlier, a key goal for ongoing and future studies will be to develop therapeutic modalities that can be implemented rapidly and safely irrespective of the type of stroke being treated. In this regard, it is noteworthy that preliminary data from our laboratory indicate that TSC also produces beneficial outcomes in an experimental model of intracranial hemorrhage [58]. It is therefore plausible that metabolic reflow therapy could be effective in treating both occlusive and hemorrhagic strokes. If so, this would obviate the need for a differential diagnosis of stroke type prior to initiating TSC treatment. Another key issue regarding the potential utility of metabolic reflow therapy concerns the time frame over which TSC can be effective. The current studies utilized a protocol in which TSC treatment was initiated soon after the onset of ischemia, which is not practicable in the context of most strokes. Ongoing studies are therefore examining the therapeutic window for TSC treatment in experimental models of stroke to ascertain whether delayed treatment is also effective in limiting ischemic damage.

In summary, our current studies have begun to characterize a novel therapeutic approach for treating ischemic neural injury. Metabolic reflow produced by TSC treatment can enhance the supply of metabolic substrates to at-risk tissue during ongoing partial ischemia. In addition, TSC treatment reduces cerebral infarction associated with permanent and temporary focal ischemia [30]. Future studies will be directed toward defining the utility of this strategy as an early intervention for the treatment of stroke, irrespective of the type of stroke that is occurring.

Conflict of interest statement We declare that we have no conflict of interest.

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