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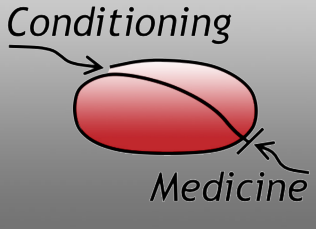

REVIEW ARTICLE | OPEN ACCESS

Help-me signaling as a paradigm for inter-cellular effects of pre- and post-conditioning in the brain after stroke

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Ischemic pre-conditioning and post-conditioning have been identified as promising therapeutic strategies for cerebral ischemia. They are based on endogenous mechanisms that the brain uses to protect itself after an ischemic event. To date, the majority of studies are focused on the acute protection of neurons per se. However, in the context of a damaged and recovering brain, all cell types in the neurovascular unit should respond to a wide range of conditioning stimuli. In this regard, the emerging concept of "help-me signaling" may be relevant, wherein injured neurons release extracellular signals that shift glial and vascular cells into potentially beneficial phenotypes. Is it possible that the beneficial effects of pre- and post-conditioning will be mediated not only by intra-cellular mechanisms within neurons, but also by the non-cell autonomous exchange of inter-cellular help-me signals between all cells in the entire neurovascular unit? In this mini-review, we propose this idea and briefly survey representative examples of this potential phenomenon, involving such molecules as chemokine (C-C) ligand 2, tumor necrosis factor alpha, vascular endothelial growth factor, and extracellular microvesicles. The ability of pre- and post-conditioning to regulate the network of help-me signals in damaged and recovering brain may offer a useful conceptual framework for future hypothesis generation and testing.

Keywords: preconditioning, postconditioning, help-me signal, neurovascular unit, neuroprotection, neurorepair, stroke

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Introduction: Promise and challenges of conditioning medicine

During ischemic stroke the brain tries to react and protect itself. However, when the insult is too severe or prolonged, these endogenous attempts usually fail. Shorter, nonlethal insults have been shown to be beneficial for their ability to produce factors that can help the brain from a future (or even recent) insult. Ischemic pre-conditioning is a neuroprotective mechanism where a small, short, nonlethal insult is able to protect the brain from a future, prolonged ischemic event. First identified in the heart, by Murry et al. in 1986 (Murry et al. 1986), pre-conditioning has since proved to be effective in the brain as well (Li et al., 2006; Kochet al., 2012). However, despite the interesting neuroprotective effect of pre-conditioning, translating this approach to the clinic is difficult because the onset of an ischemic event often cannot be predicted. For this reason, scientists have moved their attention to a more practical approach, ischemic post-conditioning, which is a series of brief non-injurious mechanical occlusions and reperfusions that may protect the brain from a recent episode of harmful ischemia (Zhao et al., 2006; Pignataro et al., 2008; Joo et al., 2013). Both strategies are thought to recruit natural adaptive responses that brain and other organs utilize to protect themselves from various insults.

In spite of a relatively large collection of promising findings, the use of cerebral ischemia as a stimulus for pre-conditioning or post-conditioning is still difficult to apply in a clinical setting (Pignataro et al., 2013). More recently, it has been suggested that conditioning and tolerance may also be stimulated with pharmacologic approaches or even by stimuli applied outside the brain, i.e. remote conditioning.

Pharmacological pre- and post-conditioning are appealing considering that most of the proposed agents are already used in clinical practice, so they are safe and well studied (i.e. opioids (Lim et al., 2004) and macrolide antibiotics (Koerner et al., 2007)). This approach carries the promise of having more direct clinical applicability. Moreover, animal studies of various pharmacological agents have showed neuroprotective effects similar to the ones extensively studied for ischemic pre-conditioning (Gidday et al., 2012; Gidday 2010). Remote ischemic pre- and post-conditioning, where the sublethal stimulus is applied to a different organ from the one injured (i.e. arm, leg etc.), have proven to be neuroprotective in both humans and rodents (Koch et al., 2011; Pignataro et al., 2013; Ren et al., 2015).

Multi-cellular effects of conditioning

To date, the majority of pre- and post-conditioning approaches have been focused on the acute protection of neurons per se. Mechanisms are mostly based on how the conditioning stimulus triggers intracellular pathways within the neuron that then serve to protect against future or recent insults. However, in the context of the neurovascular unit, it is easy to recognize that all conditioning stimuli should also affect non-neuronal cells from glial and vascular compartments. Stroke affects not only neurons but all cells in the CNS including endothelial cells, astrocytes, oligodendrocytes, microglia, and the extracellular matrix. The balance between injury and recovery will be mediated by the exchange of a wide range of released factors (Maki et al., 2013). What is required now is a rigorous understanding of how these factors can be released by various conditioning stimuli in order to help the brain protect itself. Hence, pre- and post-conditioning mechanisms may work in part by regulating an integrated help-me signaling response where specific factors are exchanged between different cells to help the entire neurovascular unit to repair and remodel (**Figure 1**).

The importance of non-cell autonomous mechanisms was recently demonstrated in a recent study showing that astrocytes may be necessary for neuronal tolerance and conditioning (Narayanan and Perez-Pinzon, 2017). During normal conditions, lactate production rates are tightly controlled. During anoxia, lactate production is increased in the brain. In the glycolytic pathway, pyruvate is decarboxylated and oxidized, producing acetyl coenzyme A and lactate. Narayanan and Perez-Pinzon (2017) showed that after ischemic preconditioning, astrocytes are able to transfer ischemic tolerance to neurons through the exchange and transfer of soluble mediators, such as lactate. Indeed, astrocytes may even transfer mitochondrial help-me signals to vulnerable neurons during ischemia (Hayakawa et al., 2016; Chou et al., 2017; Hayakawa et al., 2018). Astrocytes have long been known to play a critical supporting role for neuronal homeostasis. Perhaps it is not surprising that astrocytes may also mediate beneficial effects of brain pre- and post-conditioning.

In this mini-review, we propose the hypothesis that help-me signaling plays a vital role in non-cell autonomous modes of conditioning and tolerance for the ischemic brain. We briefly survey four representative examples of this potential phenomenon, i.e. the exchange of chemokine (C-C) ligand 2 (CCL2), tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), and extracellular vesicles, and then discuss how pre- and post-conditioning may alter the release of signals that can shift multiple cells in the neurovascular unit into beneficial phenotypes for brain repair, remodeling, and recovery.

Help-me signaling

Neurons can actively regulate other cell types. After stroke, damaged neurons are typically known to release many factors that shift glia into deleterious forms that worsen neuroinflammation. For example, after ischemia, neurons upregulate TNFα, which activates astrocytes and microglia into damaging modes that amplify neuroinflammation. Damaged neurons can also release molecules to stimulate or activate the cells around them in response to tissue injury. Traditionally, these molecules fall into broad categories of "find me" and "eat me" signals (Grimsley and Ravichandran, 2003; Napoli and Neumann, 2009). However, some of these extracellular signals may also be beneficial, and are now recognized as "help-me" signals that induce glia to adopt pro-recovery phenotypes. After injury, neurons can release mediators such as growth factors, chemokines, and cytokines that interact with receptors present on microglia to switch them into beneficial modes (Xing and Lo, 2017).

For example, it was reported that lipocalin-2 (LCN2), also known as neutrophil-gelatinase-associated-lipocalin or 24p3, a protein belonging to the lipocalin superfamily, is released by injured neurons as a help-me "distress" signal. LCN2 activates microglia and astrocytes into potentially pro-recovery phenotypes. LCN2-shifted astrocytes and microglia upregulate pro-recovery factors such as glial fibrillary acidic protein, brain derived neurotrophic factor, and interleukin-10. These LCN2-shifted cells protect neurons against oxygen-glucose deprivation and promote neuroplasticity (Xing et al., 2014). LCN2 may also contribute to brain recovery by inducing angiogenesis (Wu et al., 2015). Taken together, LCN2 may serve as a representative example of a help-me distress signal that activates microglia and astrocytes into potentially pro-recovery phenotypes.

Besides microglia, damaged neurons can also activate endothelial cells, neural stem cells, and astrocytes, providing neuroprotection and promoting neurogenesis and angiogenesis. Kyritsis et al., (2012) showed in zebrafish brain that cysteinyl leukotriene stimulates immune cells to release factors responsible for neurogenesis (Kyritsis et al., 2012).

The basic premise of help-me signaling is based on the

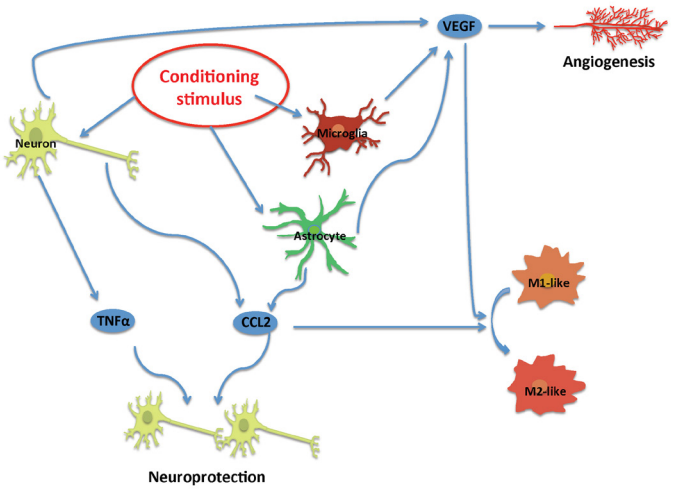

Figure 1. Schematic of pre and postconditioning and help-me signaling. Conditioning stimulates neurons, microglia, and astrocytes, altogether comprising the neurovascular unit, to release help-me signals that may shift glial and vascular cells into potentially beneficial phenotypes.

release of signals from damaged-but-not-yet-dead neurons. However, this concept can now be broadened to include the overall idea of non-cell autonomous mechanisms within the damaged and recovering brain. Besides neurons per se, many other cells in the neurovascular unit may also exchange extracellular mediators for neuroprotection and neurorecovery. Importantly, many of these mediators may be biphasic in nature. For example, VEGF can promote neural recovery by amplifying angiogenesis, but it can also be damaging by worsening blood-brain barrier leakage. So, it is extremely important to carefully dissect the balance between deleterious and beneficial effects of the neurovascular unit signaling, in order to maximize and optimize these non-cell autonomous mechanisms for neuroprotection and neurorecovery after ischemic stroke (Xing and Lo, 2017).

The basic hypothesis being proposed here states that effects of pre-conditioning and post-conditioning should involve not only neurons per se, but also affect all cells in the entire neurovascular unit. Hence, ischemic tolerance may be best understood by investigating how these approaches modify the regulation of help-me signals in the injured or diseased brain. The spectrum of help-me signals can be large, comprising cytokines, chemokines and growth factors (e.g. TNFα, CCL2, VEGF to be discussed here) that are released from all cell types from neuronal, glial and vascular compartments.

Chemokine (C-C motif) ligand 2 (CCL2)

The monocyte chemo-attractant protein1, MCP-1, also known as CCL2 belongs to the chemokine family and it is responsible for the chemo-attraction of monocytes, memory T-cells, and natural killer cells. It is constitutively expressed in neurons but also found in astrocytes, endothelium, and perivascular microglia. It binds to the CCR2 receptor on monocytes, activated T cells, and dendritic cells in the periphery, and it is expressed on microglia, astrocytes, and neurons in the brain (Conductier et al., 2010).

During stroke, CCL2 levels are increased in human blood and CSF. In animal models of stroke it increases first rapidly in neurons and later on in astrocytes. The role of CCL2 during stroke is still controversial. Some studies showed an accumulation of neutrophils and macrophages due to the overexpression of CCL2 with subsequent bigger ischemia (Wang et al., 1995; Chen et al., 2003), other studies have shown a neuroprotective effect of CCL2 with reduction of infarct size, improvement in neurological outcomes, and decrease in inflammatory markers (Strecker et al., 2011). CCL2 may have a dual role, having a detrimental effect during the acute phase of stroke by recruiting leukocytes but a protective role during the recovery phase i.e. blocking intracellular calcium increase with subsequent glutamate accumulation.

In models of hypoxic and pharmacological pre-conditioning (Wacker et al., 2012) significant cross talk was observed between hypoxia-inducible factor and sphingosine kinase 2, which together act to up-regulate CCL2 expression. Another study has shown that hypoxia-induced upregulation of CCL2 is required for ischemic tolerance. To prove this concept, CCL2-null mice were subjected to transient middle cerebral artery occlusion (MCAo) and showed no protection after ischemic pre-conditioning. Moreover, administration of a CCL2 immunoneutralizing antibody before ischemic pre-conditioning induction completely blocked the development of ischemic tolerance.

Another example of help-me signaling following pre-conditioning may be documented in peripheral nerve injury. It has been shown that neuron–macrophage interactions may activate macrophages into a pro-regenerative phenotype and that CCL2 is a key mediator of the neuron–macrophage interaction driving the pro-regenerative macrophage phenotype (Kwon et al., 2015). Also using a selective CCR-2 chemokine receptor antagonist attenuated the neuroprotective effect of both ischemic pre-conditioning, as well as post-conditioning (Rehni and Singh, 2012). However, other papers showed a reduction of CCL2 after pre-conditioning. In a model of lung ischemia-reperfusion injury, ischemic pre-conditioning significantly reduced the expression of chemokines and cytokines, including CCL2 (Rehni and Singh, 2012). In another paper, in pre- and post-ischemic myocardium, sustained ligand-activated pre-conditioning was associated with transcriptional repression of inflammation/immunity factors such as CCL2 (Ashton et al., 2013).

Tumor necrosis factor alpha (TNFα)

TNFα is a cytokine with a central role in inflammatory responses. Two main receptors bind TNFα in the brain, tumor necrosis factor receptor (TNFR) 1 (TNFR1) and TNFR2. TNFα binding to TNFR1 is mostly responsible for cell growth, cell death, and inflammation, whereas TNFR2 activates antiapoptotic and proinflammatory pathways (Aggarwal, 2003).

High levels of TNFα were found in plasma of both humans and animal models after ischemia (Vila et al., 2000; Intiso et al., 2004). In the brain, microglia is the main source for TNFα even though TNFα immunoreactivity has been shown to be present also in neurons, astrocytes, and endothelial cells (Botchkina et al., 1997). Numerous studies have shown how increases in TNFα after ischemia correlate with increases in the infarction area, and that by blocking TNFα, infarct is reduced, and outcomes are improved. However, some studies have also suggested a neuroprotective effect for TNFα, i.e. by increasing neurotrophic factors (Wilkins and Swerdlow, 2015).

Moreover, some studies have shown a protective mechanism of pre-conditioning with TNFα. Accordingly, TNFα is increased in patients with previous transient ischemic attacks. TNFα pre-conditioning significantly reduced infarct volume and inhibited microglial activation in a focal ischemia model (Nawashiro et al., 1997) and was responsible for the reduction in glutamate-induced Ca^{2+} influx in hippocampal cultures (Watters and O'Connor, 2011). Moreover, ischemic pre-conditioning up-regulated neuronal expression of TNFR1. Other studies revealed that chronic low-level exposure to TNFα, induced i.e. by exercise pre-conditioning, led to neuronal tolerance to cytokines, promoted angiogenesis, and decreased TNFα receptor expression, generating neuronal tolerance.

Furthermore, hypothermic pre-conditioning has shown to be responsible for TNFα increases in rat hippocampal cultures 24 hours after the pre-conditioning stimulus (Mitchell et al., 2011). Although the TNF mechanisms are complex and likely to be biphasic in nature, the majority of ischemic post-conditioning studies have suggested a reduction in TNFα in brain compared to controls (Hwang et al., 2017).

Vascular endothelial growth factor (VEGF)

VEGF is an important growth factor best known for promoting the formation of new vessels in endothelial cells. In humans, VEGF exists in 5 different forms, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF (the placenta growth factor). Three different receptors VEGF receptor 1 (VEGFR-1), VEGFR-2, VEGFR-3, belonging to the tyrosine kinase receptor family, can bind differentially to VEGF peptides. VEGFR-2, also known as Flk-1, binds VEGF-A, VEGF-C, and VEGF-D, and regulates vascular permeability and angiogenesis. It is now well known that VEGF, together with its effects on the vascular system, plays also a crucial role in the nervous system (Zhang and Chopp 2002).

Many studies have focused on the role of VEGF during brain ischemia. After cerebral ischemia, VEGF levels increase, peaking at 24 hours. These changes are responsible for stimulating angiogenesis and modulating vascular permeability, direct neuroprotection, and promoting neurogenesis (Ruiz de Almodovar et al., 2009). In focal stroke models, VEGF immunoreactivity can be increased in neurons, astrocytes, blood cells, and microglia. VEGF is also known to regulate neuroinflammation. It has been shown that VEGF can be released by neural progenitor cells and can regulate microglia function and activity (Mosher et al., 2012).

VEGF plays a role as mediator for help-me signaling between different elements of the neurovascular unit and it is involved in the neuroprotection achieved by pre-conditioning (Koch, et al., 2014; Park et al., 2014). Both cell culture studies and animal models have shown that after ischemic pre-conditioning, VEGF mRNA and protein expression were increased (Bernaudin et al., 2002; Li et al., 2017). The protective effects of VEGF were also confirmed in studies using anti-VEGFR1/2 monoclonal antibodies where tolerance was inhibited by blocking VEGF signaling (Laudenbach et al., 2007). Besides neurons, other cells can also release VEGF. For example, primary astrocyte cultures release VEGF and are protected from oxidative stress in response to the pre-conditioning stimulus (Chu et al., 2010). In the context of conditioning medicine, VEGF can then be exchanged as a help-me signal between multiple sources within the entire neurovascular unit.

During pre-conditioning-induced neuroprotection, VEGF is known to be increased (Park et al., 2014). As previously mentioned, it has also been shown that neural progenitor cells can release VEGF in order to regulate microglia function and activity (Mosher et al., 2012). Recently, our laboratory showed that a post-conditioning stimulus is able to increase VEGF release around the peri-infarct area, and that the interaction of VEGF with Flk-1 (VEGFR-2) on microglia activated these cells into beneficial phenotypes (Esposito et al., 2018). In this study, rats were subjected to 100 min of focal cerebral ischemia, and then randomized into a control versus post-conditioning group. As expected, 3 days after reperfusion, infarct volumes were significantly smaller in animals treated with post-conditioning, along with better neurologic outcomes. Immunostaining showed that ischemic post-conditioning increased the expression of VEGF in neurons within peri-infarct regions. Correspondingly, VEGFR-2 was expressed on Iba1-positive microglia/macrophages. Confocal microscopy showed that after post-conditioning these cells were polarized to a ramified morphology with higher expression of M2-like markers, both signs of a beneficial phenotype. Finally, treatment with a Flk-1 inhibitor to block VEGF signaling annulled the M2-like microglia polarization and negated the neuroprotection after post-conditioning. Taken together, these findings may be consistent with the role of VEGF as a "help-me signal" being released from damaged-but-not-yet-dead neurons that shifts microglia/macrophage into beneficial forms, altogether providing a neuroprotective and neurorestorative milieu in the brain (Esposito et al., 2018). Similar experiments can then be envisaged to dissect other types of help-me signals that may be affected by various modes of pre- and post-conditioning.

Exchange of microvesicles

Microvesicles, also known as microparticles, are small membrane vesicles in various mammalian cells types. They are released into the microenvironment and specifically taken up by other cells (Raposo and Stoorvogel, 2013; Colombo et al., 2014). Since microvesicles contain a large amount of RNAs and proteins, they have been increasingly recognized as important intercellular messengers, capable of responding to a variety of pathological conditions (Manuel et al., 2017; Martinez and Andriantsitohaina, 2017, Wang et al., 2017; Raeven et al., 2018), including ischemic stroke (Hayon et al., 2012; Xin et al., 2013). In a permanent MCAo model in rats, administration of platelet derived microvesicles via a biodegradable polymer to the brain surface led to a dose dependent increase in cell proliferation, neurogenesis, and angiogenesis at the infarct boundary zone, and significantly improved behavioral deficits (Hayon et al., 2012). In another study, systemic administration of mesenchymal stromal cells-generated microvesicles significantly improved neurite remodeling, neurogenesis, angiogenesis, and functional recovery after 2 hours of transient focal cerebral ischemia (Xin et al., 2013).

Recent studies have shown that microvesicles may be an especially important help-me signal for remote conditioning. Remote ischemic pre-conditioning markedly increased microvesicles released from the heart (Jeanneteau et al., 2012; Giricz et al., 2014). Microvesicles collected from coronary perfusates of donor hearts, which were subjected to ischemic pre-conditioning, exerted protective effects against ischemia/reperfusion injury in recipient isolated hearts in rats (Giricz et al., 2014). Moreover, treatment with ischemia pre-conditioning-derived microvesicles significantly alleviated the activity of caspase 3, and the expression of endoplasmic reticulum stress markers, GRP78, CHOP and caspases (Shan et al., 2013; Liu et al., 2018).

In a rat model of focal cerebral ischemia, microvesicles extracted from healthy rats that underwent hindlimb ischemia-reperfusion pre-conditioning were transfused into rats that had undergone stroke without remote ischemia pre-conditioning. The transfusion resulted in an increase in platelet-derived microvesicles in blood and reduction in infarction area, albeit to a lesser degree than remote ischemia pre-conditioning itself (Shan et al., 2013). This was the first demonstration that microvesicles are involved in the protective effect of remote ischemia pre-conditioning in a rat stroke model. However, some caution is warranted since it remains difficult to correlate microvesicle transfusion effects with any significant improvements at 24 hours or long-term.

Taken together, these studies highlight the importance of microvesicles in local and distant cell-cell communication after cerebral ischemia. Because microvesicles can travel widely throughout the body and affect multiple systems simultaneously, they may serve as logical cell-free therapeutic candidates that are triggered by remote conditioning.

Conclusions

Pre- and post-conditioning have the potential to be clinically

translated but many challenges still need to be overcome. A better knowledge of the mechanisms behind the therapeutic effects of conditioning is needed. For example, most studies in the field are focused on acute protection of neurons, whereas conditioning and tolerance will surely affect all cell types in the entire neurovascular unit. Here, we propose the hypothesis that effects of pre and post-conditioning should be investigated and interpreted in the context of help-me signaling between neural, glial, and vascular compartments in the brain. Further studies are warranted to rigorously investigate how help-me signaling is regulated in conditioning medicine.

Competing Interests

The authors declare they have no competing financial interest.

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