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Biological networks in ischemic tolerance - Rethinking the approach to clinical conditioning

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Abstract

The adaptive response (conditioning) to environmental stressors evokes evolutionarily conserved programs in uni- and multicellular organisms that result in increased fitness and resistance to stressor induced injury. Although the concept of conditioning has been around for a while, its translation into clinical therapies targeting neurovascular diseases has only recently begun. The slow pace of clinical adoption might be partially explained by our poor understanding of underpinning mechanisms and of the complex responses of the organism to the stressor. At the 2nd Translational Preconditioning Meeting participants engaged in an intense discussion addressing whether the time has come to more aggressively implement clinical conditioning protocols in the treatment of cerebrovascular diseases or whether it would be better to wait until preclinical data would help to minimize clinical empiricism. This review addresses the complex involvement of biological networks in establishing ischemic tolerance at the organism level using two clinically promising conditioning modalities, namely remote ischemic preconditioning, and per- or post-conditioning, as examples.

Introduction

Stressor-induced tolerance is a central mechanism in the response of bacteria, plants, and animals to potentially harmful changes in their environment. The response is characterized by immediate changes in cellular metabolism and by the delayed transcriptional activation or inhibition of genetic programs aimed to counter the deleterious effects of the stressor without generally being stressor specific (cross-tolerance). The immediate response is directed at preserving energy and ionic homeostasis by improving mitochondrial function, inducing translation arrest by inactivating components of the protein synthesis machinery, and by regulating the activity of ion channels and ion exchangers [1,2]. This refractory state helps the cell to survive stressor-induced environmental changes by diminishing its reliance on extracellular energy sources, metabolites and signals. By doing so, the cell becomes resistant to the stressor, but at the same time limits its own functionality by decreasing ion channel activity, inhibiting *de novo* protein synthesis, and limiting mitochondrial energy production due to partial respiratory uncoupling after K_{ATP} channel opening [3]. While these measures might be appropriate to deal with the first wave of stress, they cannot be sustained and the cell has to prepare for the case in which the stress may not subside. This is accomplished by the transcriptional activation of evolutionarily conserved protective

Conflict of Interest

The authors declare that they have no conflict of interest

programs that are reflective of the multitude of endogenous and environmental stressors we encounter. They include heat and cold shock proteins, antioxidant and detoxifying enzymes, hypoxia-responsive proteins, anti-apoptotic proteins, and components of the DNA repair machinery. The potential to harness this intrinsic ability of cells and tissues to activate these programs for therapeutic use has sparked interest among researchers and clinicians in diverse scientific fields ranging from organ transplantation to neuroscience. Because of the devastating consequences of organ ischemia/reperfusion, targeted induction of ischemic tolerance has been proposed as a primary or adjunctive therapy for vascular diseases such as stroke and myocardial infarction. In this review, we will focus on two protective paradigms, postconditioning and remote preconditioning that show promise in preclinical studies and are generally deemed adaptable for use in the clinic. Instead of concentrating on the molecular aspects of tolerance induction, which have been covered by some excellent recent reviews [4–7], we will describe system components and networks involved in inducing ischemic tolerance at the organism level and outline possible strategies for integrating the concepts obtained from animal studies into clinical settings.

Preconditioning – Definition

The term “preconditioning” has been loosely applied to many paradigms where endogenous protective pathways are activated by an exogenously applied inducer. In this review, we will refer to preconditioning as defined by Calabrese and Mattson [8,9] meaning that preconditioning is induced by exposure of cells, tissues, or whole organisms to a sublethal amount of a stressor leading to a tolerant state that confers protection to a later otherwise lethal insult. We would like to point out that the response to the stressor is bi-phasic or U-shaped in that the preconditioning effect is only observed if the stressor is applied at a certain dose range or for a certain time; increasing dose or application time above an experimentally defined threshold reverses the preconditioning effect and becomes harmful. This definition sets preconditioning apart from cytoprotective interventions that do not follow biphasic kinetics. Although the end result, namely the induction of a cytoprotected state, might be the same, the events leading to ischemic tolerance are different. Cytoprotective interventions specifically target a survival pathway without inducing severe cellular stress. For example, application of trophic factors will allow the cell or tissue to better cope with a severe insult applied at a later time by inducing pro-growth and pro-survival pathways. In contrast, preconditioning puts the cell or tissue in the precarious position of counteracting a potentially lethal stressor with an endogenous protective program that might or might not be adequate or potent enough to permit survival. Because the magnitude or even the nature of the insult is not known at the beginning, the tissue activates an array of countermeasures by invoking evolutionarily conserved pathways that try to mitigate stressor-induced changes in energy metabolism, ionic homeostasis, and redox balance. At the same time, the tissue will send system-wide signals to the organism making it systemically aware of the stressful situation and allowing other tissues or organs to activate similar programs to counter the potential for stressor effects to spread. In this regard, preconditioning is similar to other self-defense programs, for example, the immune response invoked by a local microbial infection that starts with countermeasures at the cellular and tissue level (e.g. vasodilation, inflammatory gene expression, recruitment of leukocytes) and leads to a response of the whole organism if the stressor is strong enough (e.g. fever, pain, blood leukocytosis, activation of innate immune mechanisms) finally resulting in a period of protection (i.e. adaptive immunity) against the microbial agent. Overall, the conditioning process, which results in robust increases of resistance to ischemic stress, appears in preclinical animal models to be orchestrated by broad changes in network dynamics rather than by one or a few key molecular mechanisms.

Transfer of ischemic tolerance from the periphery to the brain – mechanisms of remote ischemic conditioning (RIC)

RIC is based on the fact that transient non-injurious ischemia of one organ or tissue can protect a distant organ or tissue from ischemic injury. First described as regional preconditioning in the heart [10], soon it became evident that the concept could be applied to remote organs as well. First it was shown that brief ischemia of the kidney or small intestine was able to protect the myocardium against prolonged ischemia [11] and it has since been shown that inter-organ preconditioning can protect not only the heart, but also the intestine, kidney, liver, lung, skeletal muscle, spinal cord, and brain among others [12]. Like classical intra-organ preconditioning, RIC results in an early and delayed protective period in the target organ. RIC has attracted widespread interest, because using skeletal muscle ischemia as the RIC trigger it can be relatively easily and safely implemented in a variety of clinical settings including cardiovascular diseases and several preclinical studies show a protective effect of RIC in experimental cerebral ischemia (Table 1). The physiological and biochemical mechanisms leading to RIC involve 1) the generation of triggers and/or mediators in the distant organ or tissue undergoing transient sub-injurious ischemia, 2) release of humoral, neural or cellular transmitters, and 3) the integration of such signals in the target organ leading to ischemic tolerance (Figure 1). RIC may share mechanisms in common with other conditioning approaches such as classical local ischemic preconditioning and systemic preconditioning by application of exogenous triggers such as anesthetics or inflammatory molecules.

The response of the trigger organ to ischemia

Periods of short skeletal muscle ischemia (< 2 hours) as applied in most preconditioning protocols are unlikely to induce muscle injury [13]; however changes in vascular, myocyte, and blood leukocyte metabolism and activation status are likely. Thirty-minute ischemia of the spinotrapezius muscle in rats has been shown to increase intravascular leukocyte plugging and network resistance resulting in reduced reperfusion [14]. The same study showed an increase in xanthine oxidase-generated ROS. In humans, 20 minute forearm ischemia by inflating a blood pressure cuff resulted in an increase in neutrophil CD11b expression and platelet-neutrophil complexes in the circulating blood [15]. Apart from ROS, adenosine and bradykinin are locally generated to induce a vasodilatory response in the ischemic organ by acting on vascular smooth muscle cells and by enhancing NO and vasoactive eicosanoid production in endothelial cells, respectively. It is, however, not clear whether these trigger molecules act only locally or whether they participate as humoral factors in the preconditioning of the remote organ. Although not extensively studied, there is evidence that microRNAs can broadly influence biological processes during ischemic preconditioning in stroke models [16–18]. These small, non-coding nucleotides can post-transcriptionally modulate expression of thousands of genes [19,20] and are regulators of the biological network during stress responses. In addition, epigenetic modifications of the transcriptome that play an important role in local preconditioning (reviewed by Brand and Ratan in this issue), might also participate in RIC as it is suggested by the multi-day changes in gene expression profiles in distant organs after RIC [21]. These network regulators are likely to participate both locally and remotely in cells and tissues during the RIC process.

Humoral factors in RIC

The role of humoral factors has been directly addressed in isolated heart preparations that gained ischemic tolerance after perfusion with coronary effluent from a previously preconditioned heart [22]. Because this experimental design is generally not applicable for studying RIC in brain or spinal cord, the involvement of humoral factors in cerebral RIC has been extrapolated from aforementioned studies or was implicated by the fact that RIC could

be abolished by inhibiting adenosine or opioid receptors or by blocking NO or ROS generation during the preconditioning period [23–28].

NO/ROS: NO has been shown to mediate local ischemic and systemic inflammatory preconditioning of the brain involving neurovascular as well as direct neuroprotective mechanisms [29]. Despite a short half-life in biological fluids, NO can produce remote effects in the cardiovascular system. Red blood cells can transport NO as S-Nitroso-Hemoglobin (SNO-Hb) which is formed under high O₂ tension and might be released as NO when pO₂ drops [30]. On the other hand, because of their high reactivity and efficient catalytic enzyme systems, locally generated ROS are unlikely to act as distant signaling molecules. Nonetheless, it is conceivable that intravascular ROS alter the physiology of circulating blood cells, especially leukocytes, leading to altered reactivity and changes in their activation status that could potentially influence vascular regulation and post-ischemic inflammation in distant organs. The role of NO in RIC is disputed and NO might block RIC as shown by the reversal of hind limb ischemia-mediated cardioprotection by application of the NO donor SNAP [31]. Conversely, administration of non-selective NOS inhibitors before RIC was shown to suppress neuronal and cerebrovascular protection [24,26]. Because of the temporal proximity of RIC and index ischemia, however, a direct effect of the NOS inhibitors on the target organ cannot be excluded.

Adenosine and kininogens: Adenosine acting on A1 and A3 receptors has been implicated in several RIC models including heart [32] and brain [23]. Even though intravenous adenosine levels were increased after short periods of skeletal muscle ischemia [33], adenosine was rapidly taken up by endothelial cells and erythrocytes resulting in a plasma half-life of less than 1.5 seconds [34]. Therefore, it has been suggested that adenosine is unlikely to reach the target organ by the circulation and that instead adenosine might activate neurogenic pathways to establish RIC. One study showed that ganglionic blockade abolished the cardioprotective effect of adenosine infusion into the mesenteric artery (MA) used as a surrogate model for short term MA occlusion as a RIC inducer [32]. Similarly, adenosine infusion into the femoral artery as a way to mimic transient hind limb ischemia was effective in inducing ischemic tolerance when the dialyzed plasma of the preconditioned animal was used to perfuse an isolated heart preparation [31]. However, this protection was lost in animals where the femoral nerve was transected indicating that adenosine did not itself act as a humoral factor, but rather that it activates a neurogenic circuit that leads to the production or intravascular release of a yet unidentified humoral factor that conveys ischemic tolerance in the target organ. Similar to adenosine, local bradykinin infusion in the mesenteric circulation mimics cardiac RIC by transient MA occlusion [35]. There is strong evidence that bradykinin induces RIC through activation neurogenic pathways in ischemic and nociceptive PreC models [35,36].

Opioids and endocannabinoids: Dickson et al. demonstrated a role for opioids in the preconditioning of isolated heart preparations by perfusing the heart with the effluent from a preconditioned organ [37]. Addition of the non-specific opioid receptor (OR) antagonist naloxone to the perfusate partially blocked the protective effect, indicating that opioids might act in concert with other humoral factors to induce ischemic tolerance. Plasma levels of dynorphin, an endogenous opioid peptide signaling through the OR, were increased after hind limb ischemia and cardioprotective effects of hind limb ischemia could be blocked by a OR-selective antagonist [38]. Induction of cerebral ischemic tolerance by preceding short MA occlusion was also reverted by naloxone administration in mice [25], and hind limb ischemia induced post-conditioning was blocked by naloxone in a model of hypoxia/ischemia induced cerebral injury in neonatal rats [28]. Endocannabinoids, acting on CB1 and CB2 receptors, have been implicated in the inflammatory and NO-mediated preconditioning of the heart [39–41] and in cerebral ischemic tolerance induced by electric

acupuncture [42]. A role of cannabinoids in RIC was suggested in the cardioprotection induced by preceding short MA occlusion [43]. It was shown that RIC could be inhibited by administration of the CB2-selective antagonist AM630, but not by AM251, a selective CB1 blocker. Hind limb ischemia increased tissue levels of the endocannabinoid arachidonylethanolamide and induction of spinal ischemic tolerance was blocked by administration of AM251, but not AM630, 15 minutes before hind limb ischemia indicating the involvement of CB1 receptors in this model [44]. Microparticles from platelets, leukocytes, endothelium, and erythrocytes constitute one other potential humoral factor that may become released during the tissue stress produced by RIC and function as circulating messengers [45].

Neurogenic pathways in RIC

As a consequence of anaerobic glycolysis the lactate/pyruvate ratio increases within minutes after skeletal muscle ischemia leading to acidosis; the drop in tissue pH activates capsaicin-sensitive sensory neurons leading to activation of afferent neural pathways [46,47]. The capsaicin receptor transient receptor potential cation channel subfamily V member 1 (TRPV1) is a nonselective cation channel expressed on sensory neurons with C and A fibers. Interestingly, besides being activated by noxious stimuli such as low pH and heat, TRPV1 is also activated by the endocannabinoids, N-arachidonoyl dopamine and anandamide, hinting at a possible role of endocannabinoids functioning as neurotransmitters in establishing RIC [48,49]. Ablation of sensory nerve signaling by systemic capsaicin administration blocked RIC of the brain after hind limb ischemia [50–52]. As a result of sensory nerve stimulation calcitonin gene-related peptide (CGRP), a potent vasoactive peptide, is released and CGRP has been implicated in remote preconditioning of the brain [25]. The importance of nociceptive neural pathways in RIC induced cardioprotection has been shown by local application of capsaicin on the abdominal wall of mice 15 minutes prior to myocardial ischemia resulting in 6-fold reduction in infarct size [36].

Sensory signals from the remote organ or tissue are integrated in the CNS leading to the activation of branches of the autonomic nervous system (ANS) which conveys a protective signal to the target organ. While the role of sensory neural pathways in RIC has been substantiated, the role of efferent neural pathways in establishing ischemic tolerance is less ascertained. The role of the ANS in establishing RIC has been shown by using the nicotinic acetylcholine receptor antagonist hexamethonium bromide which acts as a ganglionic blocker of sympathetic and parasympathetic transmission. For example, ganglionic blockade reversed the cardioprotective effect of brief MA occlusion [11,32]. Moreover, vagal stimulation was sufficient to induce myocardial ischemic tolerance [53–55]. This is further corroborated by the fact that vagotomy or silencing of vagal pre-ganglionic neurons in the dorsal motor nucleus of the vagus nerve by administration of allatostatin after viral vector-mediated expression of the drosophila allatostatin receptor, resulted in reversal of cardioprotection after RIC by hind limb ischemia [56–58]. Similarly, RIC of the stomach induced by short coronary or hepatic artery occlusion was abolished after vagotomy [59]. Efferent autonomic pathways have also been implicated in tolerance against focal cerebral ischemia induced by early and delayed RIC in the rat [51,52]. Contrastingly, hexamethonium pretreatment did not abolish hind limb ischemia induced RIC in a model of skeletal muscle ischemia in the pig and of spinal cord ischemia in the rabbit, indicating that ANS involvement in establishing ischemic tolerance might not be an universal mechanism [27,60]. In conclusion, there is ample evidence for the involvement of afferent and efferent neural pathways in RIC where they function as building blocks for a “remote preconditioning reflex” as proposed by Gourine and co-workers [61]. However, the CNS structures participating in this reflex arc have not been elucidated. Furthermore,

neurohumoral coupling should also be considered and release of protective humoral factors after RIC has been shown to be dependent on sensory neural circuits [62,63].

The role of the immune system in RIC

The inflammatory response to cerebral ischemia contributes to the pathology of experimental and clinical stroke. Inflammation is initiated, sustained and possibly resolved by humoral and cellular components of the innate and adaptive immune system [64]. Several observations point to an active role of the immune system in establishing ischemic tolerance after PreC. Danger-associated molecular patterns generated and released during tissue or cellular stress are important activators of pattern recognition receptors of the innate immune system [65] and Toll-like receptors (TLRs) have been shown to reprogram the brain's response to ischemia [66]. Deletion of TLR4 abolished neuroprotection induced by ischemic PreC and mice lacking iNOS, a major proinflammatory enzyme, do not respond to ischemic PreC by BCCAO [67,68]. This would suggest that inflammatory pathways have to be, at least transiently, activated to achieve ischemic tolerance. This fact is further highlighted by the strong PreC effect of systemically administered inflammatory mediators including TNF, TLR agonists and interferons [69]. RIC induces profound changes in expression of genes associated with immune functions. Transient limb ischemia reprograms the transcriptome of human circulating leukocytes resulting in down-regulation of genes involved in TLR- and TNF-signaling, leukocyte adhesion, chemotaxis, and exocytosis while the expression of anti-inflammatory proteins such as calpastatin, heat shock protein 70 and tissue inhibitor of metalloproteinases 1 was increased [70]. Many autacoids implicated in RIC have direct effects on immune cells. For example, opioids generated locally during RIC could bind to OR or OR on circulating leukocytes resulting in heterodimerization of opioid and CCR2, CCR5 or CXCR4 chemokine receptors [71,72]. Heterodimerization of the receptors leads to desensitization and inhibition of immune cell function. Adenosine, a potent PreC trigger, by binding to A₂ and A₃ receptors on monocytes inhibits IL-12 and TNF production while increasing secretion of the anti-inflammatory cytokine IL-10 [73]. Endocannabinoids, also implicated in RIC (Table 1), are potent regulators of monocyte/macrophage and T-cell function. Activation of CB receptors suppressed IFN and IL-12 production and favored production of Th2-promoting cytokines and cannabinoids have been shown to inhibit macrophage and neutrophil migration and to suppress pro-inflammatory cytokine production in LPS stimulated microglia [74,75]. In addition to direct interactions of RIC-generated triggers and mediators with immune cells, the contributions of neural circuits to RIC-induced changes in the immune response have to be considered. The activity of the immune system is controlled by afferent and efferent neural circuits [76]. It is therefore conceivable that noxious signals generated by transient ischemia as applied during RIC activate sensory neural circuits leading to increased activation of the autonomic nervous system resulting in an altered inflammatory response after the index ischemia. Specifically, increasing evidence implicates parasympathetic signals in preserving immune homeostasis and inhibiting inflammatory responses [77]. Several experimental findings support a role for the parasympathetic nervous system in establishing cerebral ischemic tolerance. First, direct vagus nerve stimulation reduces neuronal cell death and inflammation after cerebral ischemia and suppression of parasympathetic activity, a common finding in human stroke, has been linked to increased mortality in patients with large artery atherosclerotic stroke [78–83]. Second, as described in the previous paragraph, blockage of parasympathetic signaling reverses RIC in most experimental paradigms. Third, vagus nerve-mediated cholinergic activation of 7 nicotinic acetylcholine receptors expressed on microglia and on splenic subcapsular monocytes/macrophages, which have been implicated in ischemia/reperfusion injury, has a strong immunosuppressive effect [84–86]. Taken together, there is substantial evidence for the immune system contributing to RIC by responding to local and systemic humoral factors as well as to neurogenic signals.

Per- and Post-conditioning – The clinically relevant conditioning?

Application of preconditioning protocols for the treatment of cerebrovascular diseases in humans has been hampered by the inability to predict the occurrence of an ischemic event. Because the temporal relationship of the preconditioning to the stroke may be critical for achievement of optimal stroke protection, RIPC may only be applied to a small population of patients who undergo surgical procedures that carry a risk of stroke (e.g. cardiac surgery, carotid stenting or endarterectomy) or are in a high-risk period for developing cerebral ischemia such as after subarachnoid hemorrhage. In order to apply the potential benefits of conditioning to a larger population of stroke patients other treatment modalities are needed. It has become evident that conditioning stimuli might not have to be applied before the ischemic injury, but rather could be applied during or after the index ischemia. Such phenomena have been termed per-conditioning (PerC) or post-conditioning (PostC), respectively. It was first shown that intermittent occlusion of the coronary artery during the reperfusion period (“stuttering” reperfusion) after prolonged index ischemia was as efficient as ischemic preconditioning in inducing myocardial protection [87,88]. As discussed by Dezfulian et al. and Zhao in this issue, the concept has evoked widespread interest and subsequently has not only been applied to ischemic conditioning in a variety of organs and tissues, but also to systemic chemical and inflammatory induction of ischemic tolerance (see Table 2) and might be also effective as a RIC modality (see Table 1).

PostC modalities and algorithms

Several PostC modalities have been applied to mitigate ischemia/reperfusion injury in experimental stroke and spinal cord ischemia. The majority of studies are based on the application of several cycles of intermittent ischemia during the reperfusion period after global or focal cerebral ischemia or spinal cord ischemia (Table 2). In most studies ischemic PostC was applied immediately after start of reperfusion and a narrow time window for the protection has been noted. Rabbits were protected from severe spinal cord ischemia/reperfusion injury by application of 6 postconditioning cycles 1 or 5 minutes after the start of reperfusion, but not when applied after 10 minutes [89]. In a model of combined CCA and permanent distal MCA occlusion in rats, PostC applied 10 to 30 seconds after reperfusion reduced infarct size, but not when it was initiated 3 minutes after reperfusion [90]. Rats undergoing transient MCA occlusion were protected when the MCA was transiently reoccluded after 10 minutes, but not after 30 minutes of reperfusion [91]. In a delayed model of ischemic neuronal cell death after global cerebral ischemia in rats, however, significant protection was observed when both CCAs were transiently reoccluded 48 hours after index ischemia [92–94]. Remote ischemic PostC was applied successfully after global and focal ischemia and combined ischemia/hypoxia in rats (Table 1). In remote ischemic PostC, all but one study applied hind limb ischemia at the onset of cerebral reperfusion. In the study that used delayed PostC, hind limb ischemia was successfully applied 3 and 6 hours after cerebral reperfusion [95]. Collectively the data point to the existence of more than one window of opportunity to apply PostC. So far conditioning algorithms have only been empirically established and it is likely that algorithms are specific for the model and grade of inflicted injury as shown for the myocardium and the brain [6,96].

Mediators of PostC

Like PreC, PostC can be established on the cellular, tissue, organ, and organism level [5,97]. Although deciphering the molecular mechanisms underlying PostC is just at its beginning, many triggers/mediators and effectors identified so far are shared with PreC. Adenosine acting through A2b receptors is critical for cardiac ischemic PostC [98] and administration of the opioid receptor antagonist naloxone reverted the protective effect of early ischemic

PostC after global cerebral ischemia in mice [99]. As with remote PreC, early PostC by hind limb ischemia was dependent on opioid receptor signaling in neonatal rats undergoing hypoxia/ischemia [28]. The NOS inhibitor, L-NAME, blocked early ischemic PostC in the heart and the ROS scavenger, 2-mercaptopyrionylglycine, reverted sevoflurane-induced PostC in a spinal cord ischemia model in rabbits, indicating that, similar to PreC, NO and ROS might be involved in mediating PostC [100,101]. There is also evidence for an active role of the immune system in PostC. Inhibition of the receptor CCR2, which binds the chemokine CCL2/MCP-1 and is highly expressed on certain monocyte/macrophage subsets, reverted the effect of early local ischemic PostC after global cerebral ischemia in mice suggesting that inflammatory signaling might play a role in this model [102]. It is noteworthy that RIC and PostC might also protect in animal models where white matter damage is prevalent such as neonatal hypoxia/ischemia, spinal cord ischemia, and endotoxin-induced white matter injury [27,103–107]. This is the more important, as ischemic lesions in humans preferentially affect white matter [108].

Clinical application of local or remote Pre-conditioning, Post-conditioning, or Per-conditioning

There is a wave of enthusiasm for moving directly into stroke clinical trials with one or more forms of ischemic preconditioning or a form of cross-conditioning, but some cautions and considerations would be appropriate to introduce into this rapidly developing consensus. Over the past 30 years, increasingly sophisticated molecular and cellular techniques have been applied to mechanistic studies in preclinical stroke models. Reductionist approaches in these models have greatly enriched the understanding within the stroke field of the vast numbers of cytotoxic and cytoprotective mechanisms that operate during brain ischemia (reviewed in: [109,110]). A number of these mechanisms have been postulated over the years to have a sufficiently large role in the progression of ischemic brain damage to be targeted by appropriate interventions and translated into clinical stroke treatment trials. From more than 1000 experimental treatments in preclinical models of focal brain ischemia, about 160 drug treatments had been advanced into the clinical stroke arena by 2007 [111,112]. Despite the importance of developing a cytoprotectant for stroke, virtually all efforts to translate agents that target a cellular mechanism shown preclinically to promote cell survival or to inhibit cell death have failed in clinical trials while strokes continue to occur in the United States at an average rate of about one every 40 seconds [113]. There have been many thoughtful analyses of the reasons for this failure to translate dilemma and a variety of inferences have been put forward [111,114–117]. Nonetheless, as the number of new mechanisms has progressively expanded and the number of failed clinical trials targeting single mechanisms has mounted, ischemic brain damage has gradually become more widely viewed as a highly complex, multifactorial process that involves the interplay of many non-dominant effectors in an interwoven dynamic network that in concert is very powerful [118–121]. Ongoing genomic and proteomic studies and nascent systems biology studies in an increasing number of laboratories further reinforce the same view [122–125]. Eliminating, while the brain is still salvageable, the critical hypoperfusion that drives all of these injury mechanisms has been demonstrated to have therapeutic efficacy in clinical strokes [126]. Beyond this proximate cause of ischemic brain damage, it is hard to identify a network component that can compare with insulin in diabetes or the infectious agent in pneumonia as a dominant therapeutic target. How then can one contend with such a problem? It is arguable that stroke translational research planning should incorporate some changes in the experimental approach to avoid the "trail of tears" that has characterized all prior efforts to develop effective cytoprotective therapies for stroke. The various forms of ischemic conditioning and cross-conditioning present several potential opportunities. Stress conditioning induces system-wide modifications of network dynamics that are clearly

multimodal and plurifunctional [127]. Also, as described above, conditioning of a non-CNS target organ has remote beneficial effects in brain ischemia so neuroscientists can gain potentially helpful clues about fundamental preconditioning mechanisms by examining the observations and insights of scientists focused on induction of tolerance in other organs. Development of stress conditioning therapies might be separable from the previous failures by framing the primary aim in clinical trials as the elucidation of the human network dynamics that induce tolerance to brain ischemia at transcriptomic, proteomic, and metabolomic levels. Investigators could emphasize to NIH study sections that the proposed clinical studies represent a paradigm shift in that projects of this sort have, as their ultimate goal, the definition of changes in network dynamics that preserve homeostasis under stress (i.e. tolerance) instead of attempting to identify and modulate one or more “dominant” molecular targets that are hoped to determine stroke outcomes. It will be necessary to identify and to validate one or more biomarkers that reliably indicate in preclinical models and clinical trials whether or not the selected form of conditioning has actually induced a state of tolerance (see Koch, Bahjat et al. and Wang et al. in this issue for detailed discussion). Such biomarkers would be used to separate subjects that did and did not achieve tolerance in response to the selected conditioning paradigms. A decision would have to be made whether conditioning would be a stand-alone therapeutic approach or part of a therapeutic regimen. If the latter, preliminary work would need to show that the combination did not introduce toxicity or interfere with efficacy (for example with tPA [128]). In addition to several preconditioning paradigms, a consortium could undertake several different clinical conditions with a high probability of brain ischemia in which the preconditioning paradigms would be studied (e.g. SAH, cardiac surgery, carotid endarterectomy). The team would have to include scientists who are skilled in the relevant high throughput analyses, computational biologists, statisticians, and bioinformatics specialists, in addition to basic and clinical neuroscientists. Samples such as blood that can be obtained repeatedly from patients could be collected prior to the conditioning exposure, serially after conditioning, and serially after brain ischemia. Analyses of the networks from the serial states or contexts of the various studies would permit initial modeling of a dynamic interactome that regulates the development of tolerance in humans [129]. As the network dynamics subserving tolerance become better known, efforts could be made to identify ways to boost these changes in network function. If, during the course of these studies robust clinical effects are seen, preconditioning or postconditioning could be further tested in acute strokes while efforts to learn how to boost beneficial changes in network dynamics were proceeding. An approach like this would be ground breaking and somewhat high risk so achieving funding would likely be difficult, but it would be interesting to think carefully about the potential for these or other related clinical trial designs.

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Figure 1. System networks in remote ischemic preconditioning

Trigger ischemia in the remote tissue (upper arm) induces the generation and/or release of triggers and mediators that act locally by activating sensory neurons that signal to CNS centers. Alternatively, they may function as humoral factors directly on the target organ (brain) to induce ischemic tolerance or by regulating other organ systems involved in ischemic tolerance (vasculature, immune system). Afferent neural signals are integrated and evoke the activation of efferent neurohumoral pathways that could directly alter the response of the brain tissue to index ischemia or evoke systemic effects on the vasculature (increased NO production) and the immune system (immunomodulating and down-regulating its activity) that feed back to the ischemic brain and participate in the neuroprotective response. The response is characterized by improved neurovascular function, resistance to

excitotoxicity and proapoptotic signals, reduced post-ischemic inflammation, and increased production of trophic factors involved in tissue repair.

Table 1

Remote ischemic conditioning in experimental cerebral or spinal cord ischemia

Species	Gender	RIC model	RIC site	RIC algorithm	Index Injury	Endpoint	Effect	Proposed mechanism	Ref
Neonatal rat	M/F	Pre (Immediate)	Non-invasive unilateral limb ischemia	3 x 5 m	I/H	Infarct volume, 7d	Infarct vol -70%	ND	[130]
Rat	M	Pre (-48h)	Bilateral limb ischemia	1 x 15 m 1 x 30 m	ACA	CA1 neuronal death, 7d	Neuronal death - 40%	ND	[131]
Rat	M	Pre (-1h)	Non-invasive unilateral limb ischemia	3 x 5 m	tMCAo	Infarct volume, 24h NDS, 24h	Infarct vol -50%	AIR	[23]
Rat	M	Pre (Immediate)	Invas. unilateral limb ischemia	3 x 15 m	pMCAo	Infarct volume, 48h and 60d	Infarct vol -40 to -70%	afferent sensory nerve, ANS	[52]
Rat	M	Pre (Immediate)	Invas. bilateral limb ischemia	3 x 10 m	BCCAo	Histopathology, 7d	Neuronal death - 70%	NO	[24]
Rat	M	Pre (Immediate)	Invas. bilateral limb ischemia	3 x 10 m	BCCAo	Histopathology, 7d	Reduced neuronal TUNEL	ND	[132]
Rat	M	Pre (Immediate)	Invas. bilateral limb ischemia	3 x 10 m	BCCAo	Histopathology, 7d	Significant protection	ERK1/2	[133]
Rat	M	Pre (-24h)	Non-invasive unilateral limb ischemia	5 x 5 m	BCCAo	Histopathology, 7d	No effect		[134]
Rat	M	Pre (-30m)	Invas. unilateral limb ischemia	3 x 3 m	SCI	NDS, 24h and 48h Histopathology, 48h	Neuronal death - 50%	CB1 receptors	[44]
Rat	M	Pre (-24, -48, -72h)	Invas. bilateral limb ischemia	3 x 10 m	tMCAo	Infarct volume, 24h NDS, 24h	Infarct vol -24h, -40% no effect for -48h and -72h	afferent sensory nerve, ANS	[51]
Rat	M	Pre (0, -12h, -48h)	Invas. unilateral limb ischemia	2 x 5 m 2 x 15 m 3 x 15 m	tMCAo	Infarct volume, 48h	Infarct vol (3 x 15 m RIC) 0h, -80% 12h, -50% 48h, -50%	ND	[135]
Rat	M	Pre (-15m, -48h)	Invas. bilateral limb ischemia	1 x 15 m	BCCAo	CBF (0, 5, 120m after reperfusion) Brain water content, 120m	Increased CBF, Reduced brain water content	NO	[26]
Mouse	M	Pre (Immediate)	MAo	1 x 15 m	BCCAo	Infarct volume, 24h NDS, 24h	Infarct vol -40%	mK _{ATP} channel	[136]
Mouse	M	Pre (Immediate)	MAo	1 x 15 m	BCCAo	Infarct volume, 24h; NDS, 24h	Infarct vol -45%	Insulin, opioid receptors, CGRP	[25]
Pig	NS	Pre (Immediate)	Non-invasive unilateral limb ischemia	4 x 5 m	HCA	Cerebral metabolism, 0-8h; NDS, Histopathology, 7d	Reduced pathology score	ND	[137]

Species	Gender	RIC model	RIC site	RIC algorithm	Index Injury	Endpoint	Effect	Proposed mechanism	Ref
Pig	F	Pre (-90m)	Non-invasive unilateral limb ischemia	5 x 5 m	HCA	Cerebral metabolism, 0-8h Histopathology, 24h	Better EEG, pathology score not affected	ND	[138]
Rabbit	M	Pre (Immediate)	Unilateral renal artery occlusion	2 x 5 m	SCI	NDS, 48h Histopathology, 48h	Neuronal death - 60%	ND	[139]
Rabbit	M	Pre (-30m)	Invas. bilateral limb ischemia	2 x 10 m	SCI	NDS, 48h Histopathology, 48h	Neuronal death significantly reduced	ROS, ANS not involved	[27]
Neonatal rat	M/F	Post (Immediate)	Invas. bilateral limb ischemia	4 x 10 m	I/H	Infarct vol, 48h NDS, Brain atrophy, 4 wks	Infarct vol -30%	Opioids, AKT	[28]
Rat	M	Post (+1h)	Bilateral limb ischemia	3 x 10 m	ICH	Hemorrhagic volume, BBB disruption, 72h NDS, 24 and 72h	No effect		[140]
Rat	M	Post (+3h, +6h)	Bilateral limb ischemia	3 x 5 m	tMCAo	Infarct volume, NDS 72h	Infarct vol -45%	mK _{ATP} channel	[95]
Rat	M	Post (Immediate)	Invas. bilateral limb ischemia	3 x 10 m	tMCAo	Infarct volume, BBB disruption 24h	Infarct vol -66%	ND	[141]
Rat	M	Post (immediate, +3h, +6h)	Invas. unilateral limb ischemia	3 x 15 m	pMCAo	Infarct volume, NDS 48h	Infarct vol -40% to -65%	afferent sensory nerve, protein synthesis dependent	[50]
Rat	M	Post (immediate)	Invas. bilateral limb ischemia	3 x 15 m	BCCAo	Histopathology, 7d Water maze	Neuronal death -50%	NO PI3K/Akt	[142]
Rat	M	Post (Immediate)	Invas. unilateral limb ischemia	3 x 10 m	tMCAo	Infarct volume, NDS 72h	Infarct vol -40%	ROS	[143]

AIR, adenosine A1 receptor; ACA, asphyxial cardiac arrest; ANS, autonomic nervous system; BBB, blood-brain barrier; BCCAo, bilateral carotid artery occlusion; CB1, cannabinoid receptor 1; CBF, cerebral blood flow; CGRP, Calcitonin gene related peptide; EEG, electroencephalogram; ERK, extracellular signal - regulated kinase; HCA, hypothermic circulatory arrest; I/H, ischemia/hypoxia; ICH, intracerebral hemorrhage; MAo, mesenteric artery occlusion; ND, not determined; NDS, neurological deficit score; NO, nitric oxide; NS, not specified; PI3K, phosphatidylinositol-3-OH kinase; ROS, reactive oxygen species; SCI, Spinal cord ischemia; tMCAo, transient middle cerebral artery occlusion;

Table 2

Post-conditioning in experimental cerebral or spinal cord ischemia

Class	Species	Gender	Route	Agent/ Method	Timing (rel. to R)	Index Injury	Endpoint	Effect	Proposed mechanism	Ref
Anesthetic	Rabbit	M	Systemic	Sevoflurane, 10m	0 m	SCI	Histopathology NDS 6, 24, 48h	Neuronal cell death –50%	ROS	[101]
Anesthetic	Rat	M	Systemic	Sevoflurane, 1h	0 m	pMCAo	Infarct volume 24h	Infarct volume –35%	PI3K/Akt	[144]
Anesthetic	Rat	M	Systemic	Sevoflurane, 15m	0, 5 m	tMCAo	Infarct volume 24h and 7d NDS 24h and 7d	Infarct volume 24h –44%	mK _{ATP} channel	[145]
Anesthetic	Rat	M	Systemic	Isoflurane, 30m	0 m	tMCAo	Infarct volume 24h, NDS 24h	Infarct volume –35%	ND	[146]
Anesthetic	Rat	M	Systemic	Sevoflurane, 30m	0 m	tMCAo	Infarct volume, NDS, water maze 24h	Infarct volume –40%	PI3K/Akt	[147]
Anesthetic	Rat	M	Systemic	Sevoflurane, 30m	0 m	tMCAo	Infarct volume 72h	Infarct volume –60%	ND	[148]
Anesthetic	Rat	M	Systemic	Sevoflurane 15, 30, 60m	0 m	tMCAo/ hypotension	Histopathology, NDS 72h	No effect		[149]
Chemical	Rat	M	Systemic	NE	2d	BCCAo	Histopathology, 7d	Neuronal cell death –70%	ND	[92]
Chemical	Rat	M	Systemic	3-NP	2d	BCCAo	Histopathology, 7d	Neuronal cell death –70%	ND	[92]
Chemical	Rat	M	Systemic	Bradykinin	2d	BCCAo	Histopathology, 3d	Neuronal cell death –60%	ND	[150] [151]
Chemical	Rat	M	Systemic	Kainate	0, 24, 48 h	BCCAo (2-VO)	LTP, 72h	Improved LTP (48h)	ND	[152]
Chemical	Rat	M	Systemic	Xenon, 1h	0 m	SCI	Histopathology NDS 4, 24, 48h	Neuronal cell death –20%	ND	[153]
Hypoxic	Mouse	M	Systemic	CIH	1, 5 d	tMCAo	Infarct volume 43d, NDS 1 - 28d	NDS: No effect Infarct vol.: less thalamic lesions	ND	[154]
Hypoxic	Rat	M	Systemic	Hypoxia (8% O ₂) 2 h	1 d	BCCAo	Histopathology 7d	Neuronal cell death –50%	PI3K/Akt MEK/ERK not involved	[155]
Hypoxic	Rat	M	Systemic	Hypoxia (10% O ₂) 3 x 2 h	3, 24 h	Hypoxia (5% O ₂)	Histopathology, NDS 7d	Significant protection	ND	[156]
Inflammatory	Rat	M	Systemic	LPS	2 h	SCI _{In}	Histopathology, 12h	Reduced leukocyte recruitment	ND	[157]
Ischemic	Mouse	M	Local	BCCAo, 3 x 10 s	0 m	BCCAo	Infarct volume, NDS, water maze 24h	Infarct volume –25%	NCX	[158]
Ischemic	Mouse	M	Local	BCCAo, 3 x 10 s	0 m	BCCAo	Infarct volume, NDS 24h	Infarct volume –40%	mK _{ATP} channel, Opioid receptor	[99]
Ischemic	Mouse	M	Local	BCCAo, 3 x 10 s	0 m	BCCAo	Infarct volume, NDS, water maze 24h	Infarct volume –40%	CCR2	[102]
Ischemic	Mouse	M	Local	BCCAo, 5 x 10 s	0 m	BCCAo	Infarct volume, NDS, water maze 24h	Infarct volume –50%	NP-B	[159]
Ischemic	Mouse	M	Local	BCCAo, 3 x 10 s	0 m	BCCAo	NDS, water maze 24h	NDS improved	PI3K/Akt	[160]
Ischemic	Rabbit	M	Local	AOC 3 x 3 m	0 m	SCI	Histopathology NDS 6, 24, 48h	better pathology score	ND	[103]
Ischemic	Rabbit	M	Local	AOC 5 x 1 m, Gradual reperfusion	0 m	SCI	Histopathology 28d NDS 1, 3, 7, 28d	Neuronal cell death –50%	PI3K/Akt MEK/ERK	[104]
Ischemic	Rabbit	NS	Local	AOC 6 x 1 m	0, 5, 10 m	SCI	Histopathology 10d NDS 1, 2, 5, 10d	Protection at 0 and 5 m	ND	[89]

Class	Species	Gender	Route	Agent/ Method	Timing (rel. to R)	Index Injury	Endpoint	Effect	Proposed mechanism	Ref
Ischemic	Rat	M	Local	BCCAo, 5 m	2 d	BCCAo	Histopathology, 7d	Neuronal cell death –70%	ND	[92]
Ischemic	Rat	M	Local	BCCAo, 6 x 10 s	0 m	BCCAo	Histopathology, 3d	Neuronal cell death –50%	ND	[161]
Ischemic	Rat	M	Local	BCCAo, 5 m	2 d	BCCAo	Histopathology, 7d Water maze, 7d	Neuronal cell death –85%	ROS	[93]
Ischemic	Rat	M	Local	BCCAo 3 x 15 s, 3 x 30 s	0 m	BCCAo	Histopathology 7, 21d Water maze 1 – 5d	Neuronal cell death –50%	ND	[162]
Ischemic	Rat	M	Local	BCCAo 6 x 10 s	0 m	BCCAo	Histopathology 72h	Neuronal apoptosis –80%	ND	[163]
Ischemic	Rat	M	Local	BCCAo 6 x 10 s	0 m	BCCAo	Histopathology 72h	Neuronal cell death –50%	ND	[164]
Ischemic	Rat	M	Local	BCCAo 1 x 3m	48 h	BCCAo	Histopathology 5d	Neuronal cell death –65%	PI3K/Akt	[94]
Ischemic	Rat	M	Local	BCCAo, 3 x 30 s, 3 x 10 s, 10 x 10 s	0 m	pMCAo	Infarct volume 48h,	Infarct volume –55% (at 10 x 10s PostC)	ND	[90]
Ischemic	Rat	M	Local	Uni- or bilateral CCAo, 10 s – 30 m, 1–10 cycles	0 m	pMCAo	Infarct volume 48h,	Infarct volume –50%	ND	[165]
Ischemic	Rat	M	Local	BCCAo, 3 x 10 s	0 m	pMCAo	Infarct volume, NDS 24h	Infarct volume –60%	PI3K/Akt	[166]
Ischemic	Rat	M	Local	BCCAo Stuttering reperfusion	0 m	pMCAo	Infarct volume 48h	Infarct volume –80%	ND	[96]
Ischemic	Rat	M	Local	MCAo 3 x 30 s	0 m	tMCAo	Malonyldialdeh yde levels, 24h	Reduced lipid peroxidation	ND	[167]
Ischemic	Rat	M	Local	Left CCAo, 3 x 30 s	0, 5 m	tMCAo	Infarct volume, NDS 24h	No effect		[168]
Ischemic	Rat	M	Local	MCAo 1 x 10 m	0 m	tMCAo	Infarct volume 24h	Infarct volume –60%	PI3K/Akt NCX3	[169]
Ischemic	Rat	M	Local	MCAo 5 x 2 m, 3 x 5 m, 1 x 10 m	2, 5, 10, 30 m	tMCAo	Infarct volume 24h	Infarct volume –65% (1x10m)	PI3K/Akt, ERK and p38/ MAPK not involved	[91]
Ischemic	Rat	M	Local	Right CCAo, 3 x 30 s	0, 5 m	tMCAo	Infarct volume, NDS 24h	Infarct volume –40% (0 m)	mK _{ATP} channel	[170]
Ischemic	Rat	M	Local	MCAo 3 x 15 s	0 m	tMCAo	Infarct volume 72h	Infarct volume –50%	Inhibition of MPTP opening	[171]
Ischemic	Rat	M	Local	MCAo, 3 x 30 s	0 m	tMCAo	Infarct volume, NDS 24h	Infarct volume –50%	ND	[172]
Ischemic	Rat	M	Local	MCAo, 6 x 30 s	0 m	tMCAo	Infarct volume, NDS 24h and 72h	Infarct volume –15%	ND	[173]
Ischemic	Rat	M	Local	MCAo, 6 x 30 s	0 m	tMCAo	Infarct volume, NDS 24h	Infarct volume –20%	ND	[174]

3-NP, 3-nitropropionic acid; ANS, autonomic nervous system; AOC, aortic occlusion; BCCAo, bilateral carotid artery occlusion; CCAo, common carotid artery occlusion; CGRP, Calcitonin gene related peptide; CIH, chronic intermittent hypoxia; ERK, extracellular signal – regulated kinase; MCAo, middle cerebral artery occlusion; MEK, MAP kinase or ERK kinase; MPTP , mitochondrial permeability transition pore; NCX3, sodium calcium exchanger 3; ND, not determined; NDS, neurological deficit score; NE, norepinephrine; p38/MAPK, p38 mitogen activated protein kinase; PI3K, phosphatidylinositol-3-OH kinase; pMCAo, permanent middle cerebral artery occlusion; R, reperfusion; ROS, reactive oxygen species; SCI, spinal cord injury; tMCAo, transient middle cerebral artery occlusion.