The Protective Effects of Ischemic Postconditioning against Stroke: From Rapid to Delayed and Remote Postconditioning

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Abstract: The author reviews the protective effects of ischemic postconditioning, a recently emerging strategy with broad implications in the search for new treatments in stroke and myocardial ischemic injury. Ischemic postconditioning, which refers to a series of brief ischemia and reperfusion cycles applied immediately at the site of the ischemic organ after reperfusion, results in reduced infarction in both cerebral and myocardial ischemia. Conventional postconditioning induced within a few minutes after reperfusion is arbitrarily defined as rapid postconditioning. In contrast, postconditioning performed hours to days after stroke is defined as delayed postconditioning. In addition, postconditioning can be mimicked using anesthetics or other pharmacological agents as stimuli to protect against ischemia/reperfusion injury or performed in a distant organ, which is known as remote postconditioning. In this article, the author discusses the conceptual origin of classical rapid ischemic postconditioning and its evolution into a term that represents a broad range of stimuli or triggers, including delayed postconditioning, pharmacological postconditioning, and remote postconditioning. Thereafter, various *in vivo* and *in vitro* models of postconditioning and its potential protective mechanisms are discussed. Since the concept of postconditioning is so closely associated with that of preconditioning and both share some common protective mechanisms, whether a combination of preconditioning and postconditioning offers greater protection than preconditioning or postconditioning alone is also discussed.

Keywords: Postconditioning, preconditioning, stroke, cerebral ischemia, focal ischemia, neuroprotection.

INTRODUCTION

Stroke is one of the leading causes of human disability and mortality in the world. Although extensive studies have shown that many neuroprotectants reduce infarction and improve neurological functioning in animal models of stroke, few neuroprotectants have been successfully translated into clinical applications [1]. This situation necessitates the exploration of novel neuroprotectants, such as ischemia postconditioning [2]. Ischemic postconditioning initially refers to a series of brief ischemia and reperfusion cycles applied after reperfusion, which is a concept defined in contrast with ischemic preconditioning. While postconditioning is performed after reperfusion, preconditioning is conducted before the onset of ischemia. In stroke research, the methods used for postconditioning include a stuttering reperfusion, meaning a series of intermittent hypoxia, a single period of ischemia or hypoxia, or the application of anesthetics (isoflurane) [3]. In addition, postconditioning can also be induced by the injection of certain neurotoxic agents [4]. Postconditioning can be divided into rapid and delayed postconditioning; the former refers to those applied immediately after reperfusion, and the latter are those induced a few hours to days later [3]. Postconditioning can even be executed *via* a remote organ, such as limbs; a phenomenon termed as remote postconditioning [5]. Postconditioning's protection is determined by the onset time, the duration of each occlusion or reperfusion, the number of cycles for ischemic postconditioning, the dosages of pharmacological agents, and the ischemic severity. Rapid ischemic postconditioning protects brain injury by interrupting early reperfusion, thus inhibiting free radical formation and apoptosis. Several cell signaling pathways are associated with the protective mechanisms of postconditioning, including the Akt, MAPK, PKC pathways and K_{ATP} channels. In the case of the Akt pathway and K_{ATP} channels, their inhibition abolishes the protective effects of postconditioning, whereas proteins in the MAPK and PKC pathways are modulated by postconditioning; however, whether they play a critical role requires further study. Finally, both postconditioning and preconditioning share some common protective mechanisms, but there is no evidence that the combination of preconditioning and postconditioning offers synergistic protection.

THE EVOLUTIONARY CONCEPTS FROM ISCHEMIC PRECONDITIONING TO POSTCONDITIONING

Preconditioning has served as a powerful tool for understanding the endogenous mechanisms by which the ischemic organs are protected [6]. It refers to a brief ischemia that does not cause injury to the ischemic organ and prevents ischemic injury caused by a subsequent, prolonged ischemia [6, 7]. The protective effects of ischemic preconditioning against myocardial ischemia have been studied for more than 20 years [7]. It is one of the most robust protectants identified to date against ischemia

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occurring in both the heart and brain. Its clinical application, however, has been disappointing because preconditioning can only be applied to clinical cases where an occurrence of ischemia is predictable.

In contrast to ischemic preconditioning, ischemic postconditioning is a relatively novel concept [2] that was adopted in order to contrast it with ischemic preconditioning. As such, ischemic postconditioning was believed to be a brand new concept when the term was introduced within a 6-7 year span in the research on myocardial ischemia [8, 9]. It was also believed to be derived from the concept of ischemic preconditioning [10], or from partial or controlled reperfusion [9]. However, more than 50 years ago Sewell and colleagues [11] first reported on the protective effects of intermittent reperfusion in dogs with temporary coronary arterial occlusion where ventricular fibrillation occurred within a few to 20 seconds after sudden reperfusion. They found that intermittent reperfusion, which equates to the current concept of ischemic postconditioning, abolished fibrillation [11]. In addition, this protection was once observed in a clinical case reported in 1994 where a patient was subjected to an acute myocardial ischemia [12]. Moreover, this protective phenomenon was repeated by Na and colleagues in 1996 [13], who found that postconditioning was as effective as preconditioning in preventing ventricular fibrillations in cats.

However, only after Z.Q. Zhao and colleagues published their first study on ischemic postconditioning in a myocardial ischemic model [2] have postconditioning studies thrived. In their first study, they compared the protective effects of postconditioning to preconditioning in dogs [2]. Lethal ischemia was induced by occluding the left anterior descending artery (LAD) for 60 minutes. Preconditioning was induced by applying 5 minutes of LAD occlusion with 10 minute intervals before the onset of the lethal ischemia, whereas postconditioning was conducted by 3 cycles of 30 seconds reperfusion and 30 seconds occlusion at the start of reperfusion. Infarct size was measured at 3 hours of reperfusion [2]. Their results showed that postconditioning reduced infarct size by ~44 % of the lethal ischemia, which was comparable with the protective effects of preconditioning [2]. The protective effects of postconditioning in myocardial ischemia have been confirmed by many other studies [14], including studies using models of rats [15], mice [16], rabbits [17] and pigs [18], as well as *in vitro* settings [19]. The intensive research of postconditioning in the heart has led to clinical trials. Statt *et al*. reported that postconditioning with 4 cycles of 1 minute reperfusion/1 minute occlusion *via* an angioplasty balloon reduced acute myocardial injury in patients who had ongoing myocardial infarction [20]. Taken together, the concept of ischemic postconditioning in myocardial research has been well established.

RAPID ISCHEMIC POSTCONDITIONING PROTECTS AGAINST CEREBRAL ISCHEMIA

As discussed above, ischemic postconditioning was initially defined in the field of myocardial ischemia research as a series of brief mechanical occlusions and reperfusions [2, 13]. Since then, its protective effects have been tested in other organs with ischemia/reperfusion injury, including cerebral ischemia. The mechanisms of ischemic brain injury have many similar aspects compared with those of myocardial ischemic injury. For instance, both the brain and the heart are subjected to reperfusion injury after ischemia in which free radical products play a critical role. In addition, both apoptosis and necrosis occur in the ischemic brain or heart [21-23] and similar cell signaling pathways contribute to cell death in each case. These pathways include the Akt/PKB survival pathways [24], the MAPK pathways [25], the PKC pathway [26, 27], cytochrome c/caspase mediated apoptotic pathways [28], and calpain mediated necrotic pathways [29]. Furthermore, ischemic preconditioning reduces ischemic damage in both the brain and the heart [6, 7[. Based on these similarities, it would be logical and intriguing to test whether postconditioning also protects against cerebral ischemia.

As defined above, rapid postconditioning is induced immediately or a few minutes after reperfusion, and it is the main form of postconditioning in heart research. Therefore, we and others have studied whether rapid postconditioning is effective against focal cerebral ischemia [30, 31]. In our first study we reported that rapid postconditioning reduces infarct size as a function of ischemic severity – meaning, it is less effective with longer periods of ischemia [8, 31]. We found that rapid postconditioning reduced infarct size by ~80, ~51, and ~17%, respectively, in a 15, 30, or 60 minute common carotid artery (CCA) occlusion combined with permanent distal middle cerebral artery (dMCA) occlusion. In another study we compared the impact of cycle numbers and duration of reperfusion/occlusion on the protective effect of rapid postconditioning using the ischemic model of a 30 minute bilateral CCA occlusion combined with a permanent dMCA occlusion [32]. Our results showed that rapid postconditioning conducted 10 to 30 seconds after reperfusion reduced infarct size, but not when it was initiated 3 minutes after reperfusion. Taken together, these studies suggest that the protective effects of rapid postconditioning depend on the number of cycles, the duration of each cycle of reperfusion and occlusion, and its onset time [32].

In our experiments, we observed robust protection by rapid postconditioning only in moderate or mild brain ischemia with a 15 or 30 minute bilateral CCA occlusion time [31] and mild protection in more severe ischemia (60 minutes of CCA occlusion). Xing and colleagues have also found that rapid postconditioning reduced infarction in a MCA suture occlusion model by only 16% and 12%, 1 day and 3 days after stroke, respectively [33]. Therefore, in our studies, rapid postconditioning does not appear to generate the same level of protection in the ischemic brain as has been shown in the ischemic heart [2]. However, we have not yet tested if the optimized conditions, which were defined in our recent study [32], afford better protection with a longer period of ischemia, while Xing and colleagues did not compare the protective effects with different postconditioning parameters. Therefore, we cannot exclude the possibility that the relatively weak protection is due to the usage of sub-optimal parameters (3 cycles of 30 sec reperfusion followed by 10 sec occlusion) of rapid postconditioning.

In contrast to our findings, Pignataro and colleagues demonstrated a very strong protection with postconditioning

in a severe focal ischemic model of MCA occlusion for 100 minutes [30, 34]. In their study, postconditioning with 10 minutes of occlusion initiated after 30 minutes of reperfusion offered no protection; postconditioning with 3 cycles of 5 minute reperfusion followed by 5 minute occlusion reduced infarction by 38%, and 1 cycle of 10 minute occlusion initiated after 10 minutes of reperfusion reduced infarct size by ~70 %. Again, this study suggests that the onset time of postconditioning is critical for its neuroprotective effects.

A few groups have studied the protective effects of rapid postconditioning on transient global cerebral ischemia. Rehni and Singh have shown that rapid postconditioning attenuates behavioral deficits after global ischemia in mice [35]. However, they did not report how rapid postconditioning affects neuronal loss. Nevertheless, Wang *et al*. showed that rapid postconditioning applied immediately after reperfusion attenuated neuronal death in both the hippocampus and the parietal cortex after 10 minutes of transient global ischemia [36].

The protective effects of postconditioning were further studied using *in vitro* models. One study shows that rapid postconditioning with brief oxygen glucose deprivation (OGD) blocks ischemic injury in a rat organotypic hippocampal slice culture [37]. Here, postconditioning with 3 minutes of OGD started 5 minutes after reperfusion reduced cell injury by about 40%. In addition, Pignataro *et al*. found that postconditioning with OGD reduced neuronal death in a cortical culture [30]. Postconditioning with 30 minutes of OGD conducted at 10, 30 or 60 minutes after reperfusion did not reduce cell death caused by 120 minutes OGD; however, with 10 minutes OGD initiated at 10 minutes of reperfusion, postconditioning robustly blocked cell death [30]. This study suggests that the onset time and duration of postconditioning are critical for generating neuroprotection for *in vitro* models, just as demonstrated in animal models.

DELAYED ISCHEMIC POSTCONDITIONING ATTENUATES BRAIN INJURY AFTER STROKE

In the brain, rapid ischemic postconditioning, which interrupts early reperfusion after stroke, reduces infarction. However, the extremely short therapeutic time window of rapid postconditioning, from a few seconds to minutes after reperfusion, may hinder its clinical translation. In the heart, ischemic postconditioning must be induced immediately after reperfusion to generate protection against heart injury. However, we cannot exclude the possibility that delayed postconditioning performed in the brain could be protective against brain injury. First, delayed neuronal death is usually observed after transient global ischemia. It may take 2 to 3 days for ischemic brain tissues to die, especially for those neurons in the hippocampus. Neurons in the ischemic penumbra after focal ischemia may also die in a delayed pattern. Second, like ischemic preconditioning, which has at least two therapeutic time windows (rapid preconditioning performed 1 to 3 hours before stroke onset and delayed preconditioning induced 1 to 7days before stroke onset) [6, 38, 39], postconditioning may protect against cerebral ischemia at multiple time windows. Third, ischemic postconditioning with different paradigms has different therapeutic time windows. The fact that ischemic postconditioning with certain parameters does not offer protection in the delayed time window does not exclude the possibility that postconditioning with other unidentified parameters may generate protection at later time points.

As delayed neuronal death occurs 2 to 3 days after transient global ischemia, using a transient global ischemia model is perhaps the best way to test whether delayed ischemic postconditioning offers protection. In this model, where global ischemia is induced by a 4-vessel occlusion, neuronal death is not detected at 2 days post-ischemia [40]. Using this model, Burda and colleagues found that delayed postconditioning with a single 5 minute ischemia induced 2 days after reperfusion reduced neuronal death in the hippocampus by about 94% when measured 7 days after global ischemia; a time point at which neuronal death is considered matured [40].

We recently tested the hypothesis that delayed postconditioning with different parameters reduces infarct size in focal ischemia [3]. In our study, stroke was induced by 30 minutes occlusion of the bilateral common carotid artery combined with permanent occlusion of the middle cerebral artery; the model used in our previous studies for rapid postconditioning. However, delayed postconditioning was performed not only by repetitive, brief occlusion and release of the bilateral CCAs, but also by occlusion of the ipsilateral CCA alone [3]. The results showed that delayed postconditioning performed at 3 hours and 6 hours after stroke robustly reduces infarct size, with the strongest protection achieved by delayed postconditioning with 6 cycles of 15 minutes occlusion/15 minutes release of the ipsilateral CCA executed at 6 hours. We found that this delayed postconditioning attenuated reduction in 2-[(18)F]-fluoro-2-deoxy-D-glucose (FDG)-uptake resulting in improved metabolism and reduced edema and blood brain barrier leakage. A prerequisite for performing postconditioning is that reperfusion must be achieved first; clinically, reperfusion in ischemic stroke patients is usually achieved by a tissue plasminogen activator (tPA) application. Nevertheless, t-PA may have side effects that worsen ischemic injury. Thus, we were very interested in testing whether delayed postconditioning counteracts the exacerbating effect of t-PA, and in fact, we found that delayed postconditioning reduced the infarct size increased with t-PA application [3].

We have shown that delayed postconditioning can be performed as late as 6 hours after stroke; however, increasing the delay in the therapeutic time window could potentially benefit more stroke patients who often experience delays in treatment secondary to transportation or other issues. With that said, infarction induced by focal ischemia usually matures 1 to 2 days after stroke; therefore, it seems irrational to apply delayed postconditioning later than 1 day after stroke. Nevertheless, focal ischemia may cause a secondary degeneration in the thalamus occurring a few days to a few weeks after stroke [41]. Thus, delayed postconditioning may not directly block acute brain injury, but it may attenuate the indirect or secondary brain injury induced by stroke.

Most recently, Leconte and colleagues tested an audacious hypothesis that delayed hypoxic postconditioning performed 5 days after stroke in mice, and 14 hours after

OGD in culture, protects against neuronal injury [41]. Focal ischemia was induced with 1 hour of MCA suture occlusion in mice. Delayed hypoxic postconditioning was performed by chronic intermittent hypoxia starting either 1 day or 5 days post-ischemia and continuing for 43 days when the animals were euthanized. Although both delayed postconditioning starting at 1 day or 5 days offered no protection on infarction in the ischemic region, delayed postconditioning starting at 5 days attenuated delayed thalamic atrophy when measured at 43 days. In addition, the *in vitro* study showed that hypoxic postconditioning performed 14 hours after OGD reduced neuronal death when measured 48 hours after OGD [41]. Taken together, these studies further confirm the protective effects of delayed postconditioning.

PHARMACOLOGICAL POSTCONDITIONING

Like ischemic preconditioning, a major challenge in the clinical translation of ischemic postconditioning is the high risk of directly applying an ischemic event to an organ already subjected to a severe ischemia. Other challenges include the availability of the artery that leads to ischemia for ischemic postconditioning, and whether reperfusion is achievable in a timely manner. To avoid these problems, researchers have sought drugs that can mimic the actions of ischemic postconditioning, including anesthetics or other drugs that stimulate brief ischemia in the brain, or drugs that share common mechanisms of ischemic postconditioning.

As a means to induce pharmacological postconditioning, the anesthetic isoflurane might be the best candidate to test as it has been used to induce preconditioning to protect against brain ischemia. In a suture MCA occlusion model, Lee *et al*. conducted postconditioning by maintaining 2% isoflurane for 60 minutes starting at the same time the MCA occluding suture was removed. In this study, no isoflurane was used during MCA occlusion. For rats receiving control ischemia, only 1 minute of isoflurane was used during MCA suture removal [42]. Lee *et al*. found that isoflurane postconditioning robustly reduced brain infarction and attenuated neurological deficits. They also found that rapid isoflurane postconditioning protects against ischemic injury in slice organ cultures [42], in which OGD was maintained for 15 minutes, and postconditioning was instituted by the application of isoflurane after OGD. They found that the protective effect of isoflurane postconditioning is dependent on the duration and concentration of isoflurane exposure. Lastly, isoflurane postconditioning started 0 or 10 minutes but not greater than 30 minutes post reperfusion reduced cell damage, suggesting a similar therapeutic time window with ischemic postconditioning [42].

Rapid postconditioning with brief OGD also blocks ischemic injury in rat organotypic hippocampal slice cultures [37]. Results showed that postconditioning with 3 minutes of OGD started at 5 minutes after reperfusion reduced cell injury by about 40%. In the same study, protection by postconditioning was also induced by adding a low dose of the pharmacological agent, 3, 5-dihydroxyphenylglycine (DHPG, a group 1 metabotropic glutamate receptor agonist) 5 minutes after reperfusion and incubating for 30 minutes. It has been reported that high doses of DHPG exacerbate, while low doses inhibit, neuronal injury [37].

Pharmacological treatments can also be used to mimic delayed postconditioning conducted at 2 days after reperfusion in rat global ischemia models [40]. In these studies, global ischemia was induced by 4-vessel occlusion, and pharmacological postconditioning was conducted by an injection of 3-nitropropionic acid (3-NP), norepinephrine, or bradykinin [43, 44]. Delayed postconditioning with an intraperitoneal injection of norepinephrine, bradykinin, or 3NP at 2 days resulted in increased-neuronal survival after global ischemia [40, 45]. These protections are comparable with those seen by delayed postconditioning with 5-6 minutes of ischemia [40, 45].

CONVENTIONAL ISCHEMIC POSTCONDITIONING IS EXTENDED TO REMOTE POSTCONDITIONING

As discussed above, classical pre or postconditioning is conducted in the same organ that receives prolonged ischemia [7, 38, 46, 47]. Its clinical application is limited by the risk of applying an additional ischemia to a vital organ, such as the brain or the heart. The concept of conventional ischemic pre and postconditioning has been extended not only to pharmacological pre and postconditioning, but also to remote ischemic pre and postconditioning [48]. Previous studies have shown that remote preconditioning performed in a limb [49, 50], kidney [48] or in the mesentery [51] protects against a subsequent ischemia in the heart. Similarly, many studies have shown that remote postconditioning reduces ischemic injury in the heart.

Although the protective effects of remote pre and postconditioning have been studied extensively in the research field of myocardial ischemia [52], much less is known in the field of stroke. A few studies exist regarding the protective effects of remote preconditioning against brain ischemia that demonstrate that limb ischemia reduces delayed neuronal death in the hippocampal CA 1 region [53-58]. Recently we have generated solid evidence that remote preconditioning in the limb reduces infarct size in a focal ischemia model in rats. We showed that with immediate limb ischemia, 12 hours and 48 hours before cerebral ischemia, limb preconditioning protects against focal cerebral ischemia in rats. We found that the protective effects of remote preconditioning could be induced in the single hind limb ipsilateral to the ischemic hemisphere. Based on this study in our own laboratory and other previous studies showing that remote postconditioning reduces heart injury after ischemia [59], we further tested our hypothesis that remote postconditioning conducted in the ipsilateral hind limb protects against focal ischemia [5]. As a result, we have now demonstrated that remote postconditioning conducted immediately after reperfusion markedly reduces infarct size. Stroke was generated in male rats by a permanent occlusion of the left distal middle cerebral artery combined with a 30 minute occlusion of the bilateral CCA. After CCA release, remote postconditioning was generated by 3 cycles of 15 minute occlusion followed by 15 minute release of the left hind femoral artery. We found that rapid remote postconditioning performed immediately after CCA release reduces infarction by 67% measured at 2 days after stroke. In addition, we found that delayed remote postconditioning initiated as late as 3 hours, though not 6 hours, still robustly reduces infarction by 43% 2 days after stroke [5]. These

results suggest that remote postconditioning provides a wide therapeutic time window for clinical translation.

POSTCONDITIONING OFFERS LONG-TERM PROTECTIVE EFFECTS AND IMPROVES BRAIN FUNCTION AFTER STROKE

Whether postconditioning provides lasting protection and preserves brain function is another critical issue that demands confirmation because some neuroprotectants, such as post-ischemic hypothermia [60] and rapid ischemic preconditioning [6], have been shown to provide temporary protection for only a few days after ischemia. In addition, reducing injured brain tissue may not translate into the preservation of neurological function [61].

In a long-term study, we found that rapid postconditioning performed immediately after reperfusion reduced lesion size ~40% in rats subjected to ischemia when measured 30 days after ischemia [62]. In addition, using a vibrissae test to detect asymmetrical forelimb usage, we found that rapid postconditioning improves neurological function [62]. In global ischemia rapid postconditioning also improves subject performance on spatial learning and memory in a water maze test 3 weeks after reperfusion [36], suggesting that rapid postconditioning provides long-term protection in global ischemia, which is consistent with its protective effects on neuronal survival.

We found that both rapid postconditioning and delayed postconditioning offer long-term protection and improve functional recovery. Our results showed that delayed ischemic postconditioning conducted from 6 hours after stroke attenuates brain injury and improves the outcomes of behavioral tests for up to 2 months using 4 standard testing methods, including the vibrissae test, postural reflex test, tail hang test, and home cage test [63].

In contrast, our recent study showed that remote postconditioning did not reduce infarct size measured at 2 months after stroke, although the infarction measured 2 days post-stroke was reduced, suggesting that remote preconditioning in our study only executes transient protection on infarct size. Nevertheless, we found in the same study that remote postconditioning improves the outcome of the behavioral test for up to 2 months post-ischemia. The underlying mechanisms regarding the improved neurological function without sparing infarction remain to be clarified.

A COMBINATION OF RAPID POSTCONDITIONING WITH PRECONDITIONING OFFERS NO SYNERGISTIC PROTECTION

The protective mechanisms of pre and postconditioning have been studied extensively in the heart and results suggest there are common mechanisms. For example, both pre and postconditioning protect the ischemic organ by enhancing adenosine activity, reducing the products of reactive oxygen species and lipid peroxidation [2, 64] and inhibiting c-Jun N-terminal kinase (JNK)/P38 activity [65], while promoting extracellular signal-regulated kinase 1/2 (ERK1/2) activity [66]. The above discussed mechanisms regarding postconditioning against cerebral ischemia also have similarities with those of preconditioning in cerebral ischemia. Therefore, the combination study may provide further clues to understanding the protective mechanisms of both pre and postconditioning. In addition, because both pre and postconditioning may be feasible in certain clinical settings where stroke occurrence is predictable, the combination study may help clinicians decide if a combination of both pre and postconditioning is an option for clinical translation.

Our laboratory recently compared the protective effects of rapid postconditioning combined with rapid or delayed preconditioning [32]. Rapid preconditioning was induced by transiently occluding the left dMCA for 15 minutes at 60 minutes before stroke [32], while delayed preconditioning was induced by occluding the left dMCA for 5 or 15 minutes at 3 days before the stroke. Postconditioning of 10 cycles of 10 seconds occlusion/10 seconds reperfusion was conducted immediately after reperfusion. We found that the protective effect of postconditioning is comparable with that of rapid preconditioning, but is less effective than delayed preconditioning. When addressing whether postconditioning plus preconditioning generates a synergistic effect [32], we found that postconditioning combined with either rapid or delayed preconditioning does not provide any additional reductions in infarction. Our results are consistent with Pignataro and colleagues who reported that protection with postconditioning is comparable to that of delayed preconditioning in a suture MCA occlusion model in rats, while a combination of pre and postconditioning provides no greater protection [30].

In the above combination studies, both pre and postconditioning were induced similarly using a brief ischemia, or a series of brief ischemia. While these combinations offered no synergistic protection, the possibility exists that different combinations of modalities, such as ischemic postconditioning with a hypoxic preconditioning or 3-NP preconditioning, could achieve greater protection, and further study is warranted.

PROTECTIVE MECHANISMS OF CONVENTIONAL AND REMOTE POSTCONDITIONING AGAINST STROKE

Current studies regarding the underlying protective mechanisms of postconditioning focus on rapid ischemic postconditioning. Since rapid ischemic postconditioning interrupts early reperfusion, its protective effects must be closely associated with changes in cerebral blood flow (CBF) after reperfusion and with subsequent events, such as free radical production, endothelial function, and changes in blood brain barrier (BBB) integrity and inflammation that occur due to interrupted CBF. In our studies, we first confirmed whether rapid postconditioning attenuates the hyperemic response induced by reperfusion, and whether it mitigates hypotension thereafter. CBF was measured by a Laser Doppler Probe in the penumbra in rats subjected to 15 or 30 minutes of bilateral CCA occlusion combined with permanent MCA occlusion [31, 32]. We detected a clear hyperemic response after reperfusion in rats subjected to a 15 minute occlusion, and CBF was recovered to pre-ischemic levels in rats with 30 minutes of occlusion [31, 32]. We showed that rapid postconditioning with mechanical interruption results in CBF changes, and CBF at 30 minutes

after reperfusion was improved [32]. Wang and colleagues confirmed this effect in a global ischemia model [36].

Next, we examined whether rapid postconditioning attenuates reactive oxygen species (ROS) production and apoptosis as early reperfusion is known to cause increased ROS products leading to apoptosis. We found that rapid postconditioning profoundly attenuates the amount of superoxide at 30 minutes after reperfusion in the model of 30 minutes CCA occlusion plus permanent MCA occlusion [31]. Consistent with our findings, Xing and colleagues reported that rapid postconditioning attenuates lipid peroxidase levels in a focal ischemia model [33] and Danielisova and colleagues have shown that delayed postconditioning performed 2 days after global ischemia increases activities of antioxidant enzymes, including superoxide dismutase and catalase [44]. Furthermore, we have shown that rapid postconditioning blocked terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) positive staining, a marker of apoptosis, in the penumbra 2 days after stroke [31]. Wang and colleagues further showed that rapid postconditioning reduced cytochrome c release from the mitochondria to the cytosol, a critical cascade for apoptosis induction [36]. Taken together, these data suggest that postconditioning may reduce ischemic injury by blocking ROS activities and apoptosis.

The protective effects of rapid postconditioning on inflammation after stroke have also been explored. Rapid postconditioning inhibits myeloperoxidase (MPO) activity, an indicator of leukocyte accumulation, and the expression of IL-1β and TNF-α mRNA, and ICAM-1 protein expression in the ischemic cortex at 24 hours after ischemia [33]. These results suggest that rapid postconditioning may produce an anti-inflammatory effect.

Consistent with the positive effects of rapid postconditioning on CBF, we have reported that delayed postconditioning enhances glucose uptake or metabolism as detected by micro PET imaging [63]. In addition, delayed postconditioning attenuates edema formation and BBB leakage.

Multiple pathways are involved in neuronal death after stroke, including the PKC pathways, MAPK pathways, and PI3K/Akt Pathway. These pathways contain both pro- and anti-apoptotic signals, and their respective levels of activation/inactivation decide the fate of ischemic neurons after stroke. In the PKC pathways, at least 11 isozymes of the PKC family exist, including δPKC and εPKC [67]. While δPKC activity usually leads to cell death [68], εPKC promotes neuronal survival [26]. PKC isozymes differ as to their intracellular location and function, and their activities are regulated by their cleavage form, phosphorylation, and subcellular location. We found that at 1 hour after stroke rapid postconditioning did not affect the protein levels of total δPKC in the penumbra but did block the increase in levels of the cleaved form of δPKC, indicating δPKC activity [62]. Rapid postconditioning had no effect on phosphorylated δPKC (thr 505) levels, which decreased by 24 hours after stroke onset; however, it strongly inhibited decreases in phosphorylated εPKC levels after stroke. Thus, rapid postconditioning may reduce ischemic damage by inhibiting the worsening effect of δPKC activity while promoting a beneficial effect of εPKC activity [62]. Nevertheless, more studies are needed to clarify the role of PKC pathways in the protective effects of ischemic postconditioning.

Ischemic injury and neuronal survival are modulated by the MAPK pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), P38, and c-Jun N-terminal kinase (JNK) pathways [69]. As we have reviewed, JNK and p38 are clearly detrimental after stroke, and their inhibition blocks apoptosis in many neuronal death paradigms [69]. However, ERK1/2's activity is involved in both neuroprotection as well as injury exacerbation [69]. In general, most studies agree that ERK1/2 phosphorylation is transiently increased after stroke, suggesting increases in ERK1/2 activity induced by ischemia/reperfusion. Such an increase is confounding as ERK1/2 is apparently involved in the beneficial effects of growth factors, estrogen, preconditioning, and hypothermia on the ischemic brain, but it also promotes inflammation and oxidative stress, and its inhibition reduces ischemic damage [69]. Given such controversy, we were very interested in studying the changes in ERK1/2 activity involved in the protective effects in postconditioning.

In our pilot study ERK1/2 phosphorylation (P-ERK1/2) levels increased from 1 to 24 hours after stroke, and rapid postconditioning reduced their levels in the penumbra [62]. Our results imply a detrimental role for P-ERK1/2 after ischemia; however, we did not study whether its inhibition contributes to the protection of rapid postconditioning. Our observation conflicts with Pignataro and colleagues who showed that rapid postconditioning enhances ERK1/2 phosphorylation [30] and furthermore, U0126, the antagonist of the MEK/ERK1/2 pathway, does not block the protection of rapid postconditioning. This would indicate that increased levels of P-ERK1/2 may be unrelated to the protective effect of rapid postconditioning. More detailed experiments are required to resolve the discrepancy between our results and that of Pignataro *et al*.

The Akt pathway plays a critical role in neuronal survival after stroke; Akt dysfunction results in apoptosis induction, while Akt activity blocks apoptosis by phosphorylating its substrates, including GSK3β (glycogen synthase kinase 3β), FKHR and Bad. Akt activity is considered to be regulated by phosphorylation, which is modulated by upstream molecular signals, such as PTEN and PDK1 (phosphoinositide-dependent protein kinase-1). Akt activity increases with increased phosphorylation of PTEN (*phosphatase and tensin homologue deleted on chromosome 10*) and PDK1, and GSK3β phosphorylation supports cell survival (Zhao *et al*. 2006a, review). Dephosphorylation of GSK3β leads to its activation and to phosphorylation of β-catenin, which results in β-catenin degradation and apoptosis (Zhao *et al*. 2006a). We found that rapid postconditioning increases both Akt phosphorylation (by Western blot) [30, 62] and Akt activity (by *in vitro* kinase assay) [62]. Furthermore, Akt inhibition by injection of the PI3K inhibitor, LY294002, partially blocks the protective effect of rapid postconditioning [30, 62]. However, rapid postconditioning does not affect phosphorylation of PTEN or PDK1 but it does inhibit an increase in GSK3β phosphorylation. We found that rapid postconditioning blocks β-catenin phosphorylation, but has

no effect on total or non-phosphorylated β-catenin protein levels [62]. Taken together, the Akt pathway plays a critical role in protection by postconditioning. Our results are further supported by a recent *in vitro* experiment showing that Akt inhibition abolished the protective effect of OGD and DHPG postconditioning in hippocampal slice cultures [37].

In addition, ATP-sensitive potassium channel (K_{ATP}) channels may also play a critical role in brain injury after stroke. After ischemia, ATP depletion results in the opening of K_{ATP} channels, which is required for the induction of the protective effects of ischemic preconditioning as well as postconditioning in the heart. There are 2 K_{ATP} channels, the sarcolemmal and mitochondrial, and they vary by location. The mitochondrial K_{ATP} channels have been studied the most as their opening generates an outward current that stabilizes the mitochondrial membrane and blocks cell death. In the same vein, Lee and colleagues reported that both a general channel blocker, glibenclamide, and a mitochondrial channel blocker, 5-HD, abolish the protective effect of isoflurane postconditioning, suggesting that K_{ATP} channels may be involved in the protective mechanisms of postconditioning. Compared to traditional rapid postconditioning, little is known about the underlying protective mechanisms of remote postconditioning. Nevertheless, regarding the heart, accumulating evidence suggests that neural pathways serve as a connection between the remote preconditioned organ and the heart. Wolfrum *et al*. reported that remote preconditioning with brief mesenteric artery occlusion/reperfusion reduces heart infarction by activating εPKC in rats [70]. However, this protection was blocked by pretreatment with the ganglion blocker, hexamethonium [70]. In another study, remote preconditioning resulted in bradykinin release, which stimulates sensory nerves and offers protection, and this effect is blocked by the ganglion blocker, hexamethonium [71]. In addition, inhibition of afferent nerves with capsaicin abolished the protective effects of remote preconditioning against gastric ischemia when remote preconditioning is conducted the heart or liver by 2, 5 minute ischemic occlusions of the coronal or hepatic arteries [72]. Consistent with these findings, we recently demonstrated that capsaicin treatment reverses protection by remote postconditioning, suggesting that the afferent nerve pathways may sever a connection between the remote organ, limb, and the ischemic brain[5]. We also demonstrated that cycloheximide, a protein synthesis inhibitor, robustly attenuates the protective effect of remote postconditioning, although the underlying mechanisms are unclear. Cycloheximide has been typically shown to inhibit the protective effects of preconditioning against ischemic injury by blocking de novo protein synthesis [73]. It is not surprising that a protein synthesis inhibitor blocks the protective effects of preconditioning because preconditioning is carried out a few hours to days before ischemia onset [73-75], and preconditioning may have time to stimulate the organ to adapt to a future ischemic event, including increasing protein synthesis. In the case of remote postconditioning, the brain may not have time to synthesize the new proteins required for neuroprotection because it is performed immediately after reperfusion. Why protein synthesis inhibition abolishes the protective effects of remote postconditioning remains elusive.

Taken together, previous studies have mainly focused on studying the protective mechanisms of rapid postconditioning, while little is known regarding delayed postconditioning. Since rapid postconditioning is applied immediately after reperfusion, it is able to attenuate those detrimental responses induced by reperfusion, such as free radical products, and the associated interruption of various cell signaling pathways. However, delayed postconditioning is applied a few hours, even a few days after reperfusion; it may modulate the secondary responses that occur much later after reperfusion injury. For instance, delayed postconditioning may attenuate CBF inhibition that occurs at later time points after initial reperfusion; it may also regulate inflammatory response, a relatively delayed detrimental event after stroke. Additionally, delayed postconditioning may promote angiogenesis and neurogenesis. In the future, how delayed postconditioning affects these late cascades after stroke should be studied next.

SUMMARY AND CONCLUSIONS

The protective effects of rapid ischemic postconditioning against cerebral ischemia have been well established; however, more studies are needed to confirm whether delayed postconditioning or remote postconditioning offers similar protection. Rapid postconditioning appears to act by blocking the overproduction of ROS and lipid peroxidation, and by inhibiting apoptosis. Akt and K_{ATP} channel activity contribute to its protective effects and changes in MAPK pathways and δPKC and εPKC activities also play a role. Future studies will be necessary to clarify the underlying protective mechanisms observed in delayed and remote postconditioning.

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ABBREVIATIONS

K_{ATP} channels	=	ATP-sensitive potassium channel channels
CCA	=	Common carotid artery
dMCA	=	Distal middle cerebral artery
DHPG	=	3,5-Dihydroxyphenylglycine
3-NP	=	3-Nitropropionic acid
CBF	=	Cerebral blood flow
BBB	=	Blood brain barrier
TUNEL	=	Terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling
LAD	=	Left anterior descending artery
MPO	=	Myeloperoxidase
ERK1/2	=	Extracellular signal-regulated kinase ½
JNK	=	c-Jun N-terminal kinase

PTEN = Phosphatase and tensin homologue deleted on chromosome 10

PDK1 = Phosphoinositide-dependent protein kinase-1

GSK3β = Glycogen synthase kinase 3β

ROS = Reactive oxygen species

OGD = Oxygen glucose deprivation

tPA = Tissue plasminogen activator,

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