Neuroprotective Effects of Ischemic Preconditioning in Brain Mitochondria Following Cerebral Ischemia

Miguel A. Pérez-Pinzón[1,2]

Numerous studies support the hypothesis that reperfusion following cerebral ischemia contributes substantially to ischemic injury and that mitochondrial dysfunction plays a central role. Defining the mechanisms by which mitochondrial dysfunction occurs may be important for the development of new therapies against delayed neuronal cell death. Ischemic preconditioning (IP) increases an organ's resistance to ischemic injury. There are two windows for IPC, one that requires several hours to develop and another one with a rapid setting (rapid window). However, the rapid window only provides neuroprotection for few days. We have recently determined that this lack of chronic protection by the rapid window was due to lack of protection against mitochondrial dysfunction.

KEY WORDS: Cerebral ischemia; bioenergetics; metabolism; anoxia.

The mechanisms leading to neuronal cell death after cerebral ischemia are complex. A well established fact in this field is that cells continue to die over months after a stroke, a phenomena that has been termed delayed cell death. Although not clearly defined, neuronal cell death may result from either apoptosis, necrosis, or a cell death mechanisms that is a mixture of these processes (Liou *et al.*, 2003; Martin *et al.*, 1998).

Numerous studies support the hypothesis that reperfusion following cerebral ischemia contributes substantially to ischemic injury (Chan, 1994; Choi, 1993; Siesjo and Smith, 1991; Watson and Ginsberg, 1989) and that mitochondrial dysfunction plays a central role (Abe *et al.*, 1995; Ankarcrona *et al.*, 1995; Fiskum *et al.*, 1999; Friberg and Wieloch, 2002; Schinder *et al.*, 1996; White and Reynolds, 1996, 1997).

Evidence of mitochondrial dysfunction following cerebral ischemia was described in previous studies, as a prominent change in redox activity of mitochondrial respiratory chain components in postischemic brain (Perez-Pinzon *et al.*, 1997a,b; Rosenthal *et al.*, 1995, 1997; Welsh

et al., 1982, 1991). This hyperoxidation of electron carriers is indicative of either a response to decreased substrate availability (Rosenthal *et al.*, 1995) and/or a reaction of mitochondrial complexes to reactive oxygen species (ROS) (Perez-Pinzon *et al.*, 1997b). Postischemic mitochondrial may also be a major source of ROS, and free radical-mediated damage has been linked to reperfusion injury following brain ischemia (Flamm *et al.*, 1978; Fridovich, 1979; Hall and Braughler, 1993; Kontos, 1989; Siesjo *et al.*, 1985; Vlessis *et al.*, 1990). However, recent findings suggest that this hyperoxidation may result from loss of electron carriers from mitochondria following cerebral ischemia, such as cytochrome *c* and NADH (Perez-Pinzon *et al.*, 1999c). The loss of cytochrome *c* from mitochondria might affect respiratory chain activity and/or it may trigger the apoptotic cascade (Charriaut-Marlangue *et al.*, 1996; Nitatori *et al.*, 1995). This is suggested by findings that apoptosis (programmed cell death) may be linked to mitochondria and their release of cytochrome *c* (Ankarcrona *et al.*, 1995; Kluck *et al.*, 1997; Schinder *et al.*, 1996; Yang *et al.*, 1997).

Additional evidence of mitochondrial dysfunction was described in studies from isolated brain mitochondria. Miotochondria isolated from ischemic brain exhibited decreases in state 3 respiratory rates of approximately 70% with NAD-linked respiratory substrates (Sciamanna *et al.*, 1992). Cafe *et al.* (1994) showed that nonsynaptosomal

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[1] Cerebral Vascular Disease Research Center, Department of Neurology and Neuroscience Program, University of Miami School of Medicine, Miami, Florida.

[2] To whom correspondence should be addressed at Department of Neurology (D4-5), P.O. Box 016960, University of Miami School of Medicine, Miami, Florida 33101; e-mail: perezpinzon@miami.edu.

mitochondria were insensitive to ischemia but that they became dysfunctional in the late reperfusion phase. Mitochondria from synaptic terminals were greatly affected by ischemia but partially recovered during reperfusion. Sims and Pulsinelli (1987) also reported that in rat a model of forebrain transient ischemia the rate of oxygen consumption decreased in the CA1, CA3, and CA4 regions in the late reperfusion phase. This study was performed in homogenates from different brain subregions.

ISCHEMIC PRECONDITIONING

Ischemic preconditioning (IP) refers to the ability of a brief ("sublethal") ischemic episode, followed by a period of reperfusion, to increase an organ's resistance to injury (ischemic tolerance) following a subsequent ischemic event. This induction of tolerance against ischemia resulting from sublethal ischemic or anoxic insults has gained attention as a robust neuroprotective mechanism against conditions of stress such as anoxia/ischemia in heart and brain (Alkhulaifi *et al.*, 1993; Kato *et al.*, 1992; Kitagawa *et al.*, 1990; Lin *et al.*, 1992, 1993; Murry *et al.* 1986; Walker *et al.*, 1993). There are different preconditioning paradigms both in heart and brain. Among variations in preconditioning paradigms include, the number of pre-

conditioning insults, types of preconditioning insults, and time between preconditioning and the test insults.

In the past, most preconditioning studies in brain have suggested that several hours are required to develop the tolerant state. However, in recent studies we and others suggested that preconditioning with a rapid onset time course, similar to that in heart (within 1 h), can protect synaptic activity after anoxia in brain slices (Centeno *et al.*, 1999; Perez-Pinzon *et al.*, 1996, 1999a; Perez-Pinzon and Born, 1999; Schurr *et al.*, 1986; Schurr and Rigor, 1987) and reduce histopathology after ischemia in intact brain (Atochin *et al.*, 2003; Nakamura *et al.*, 2002; Perez-Pinzon, 2000; Perez-Pinzon *et al.*, 1997c; Stagliano *et al.*, 1999).

Thus, two windows have been identified (see Fig. 1), one that occurs very rapidly (within 1 h) (Perez-Pinzon, 2000; Perez-Pinzon *et al.*, 1997c; Stagliano *et al.*, 1999) and a second one that develops slowly (over days) (Kitagawa *et al.*, 1990). A difference between these two windows is that neuroprotection in the first window is transient. We observed that significant neuroprotection against histopathology was evident after 3 days of reperfusion, but after 7 days, histopathology was similar between controls and preconditioned rats (Perez-Pinzon *et al.*, 1997c). In contrast, when rats were preconditioned days prior to the "test" ischemic insult, the neuroprotection was robust
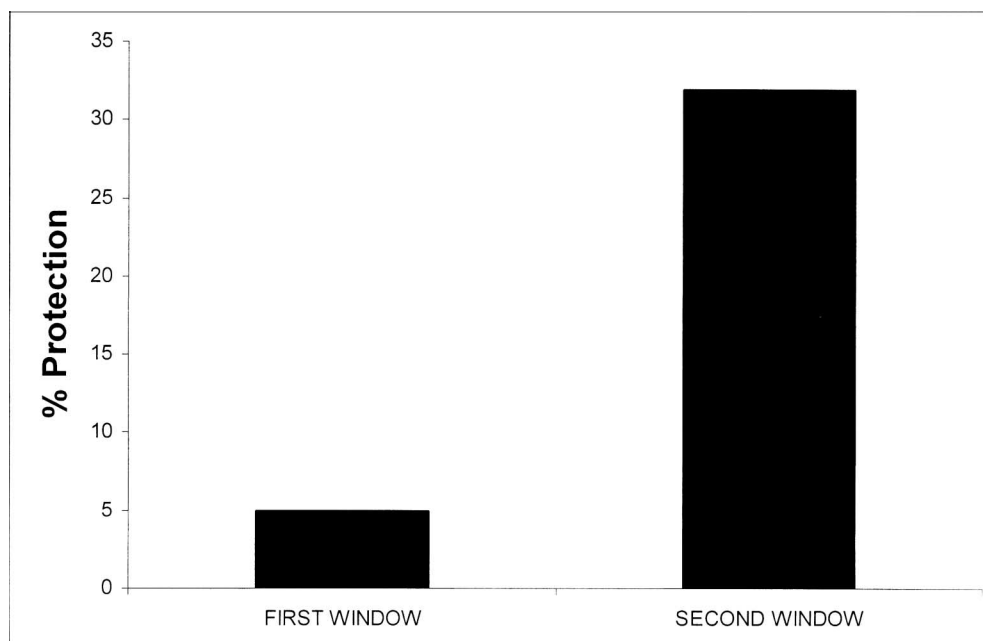

Fig. 1. Comparison between the % protection in the two windows of ischemic preconditioning. In the first window, IPC preceded the test ischemic insult by 30 min. In this window, IPC protected against histopathology at 3 days, but not 7 days of reperfusion. In contrast, when IPC preceded the test ischemic insult by 48 h, there was approximately 33% protection of normal neurons after 7 days of reperfusion.

and long lasting (Kitagawa *et al.*, 1990; Kurkinen *et al.*, 2001).

IPC and Mitochondrial Protection
in the First Window

Recent studies from this laboratory and others suggest that preconditioning from the first window can protect ischemic injury, as assessed by the release of lactic dehydrogenase (LDH) or by synaptic activity recovery after the test insults in cell cultures (Reshef *et al.*, 1996), brain slices (Centeno *et al.*, 1999; Perez-Pinzon and Born, 1999; Perez-Pinzon *et al.*, 1996, 1999a; Schurr *et al.*, 1986; Schurr and Rigor, 1987) and reduce histopathology after ischemia in intact brain (Atochin *et al.*, 2003; Nakamura *et al.*, 2002; Perez-Pinzon, 2000; Perez-Pinzon *et al.*, 1997c, 1999b). However, protection only ensued if reperfusion was allowed to occur for 3 days but not beyond (Perez-Pinzon *et al.*, 1997c). Thus, it was important to ascertain whether this lack of chronic protection was due to less protection against mitochondrial dysfunction by IPC.

Indeed, we found that the first window of IPC could not protect mitochondria against the deficits in respiration through complexes I–IV (Perez-Pinzon *et al.*, 2002). Rates of respiration in presence of pyruvate $+$ malate and succinate $+$ glycerol $- 3 -$ phosphate decreased in both the ischemia and in IPC groups marginally. However, significant decreases in the rate of respiration for complex IV were observed in both the ischemia and IPC groups. This decrease in the rate of respiration in the presence of complex IV substrates is suggestive of impairment in oxidative phosphorylation. This decrease in the rate of respiration at the level of complex IV was not accompanied by significant decreases in complex IV activity. A possible explanation for the decrease of respiration in complex IV and no change in complex IV activity in the ischemia group is that cytochrome *c* may be released from mitochondria following test ischemia. This contention is supported by previous studies. We found that the apparent mitochondrial hyperoxidation linked to brain dysfunction may be caused by disruption of the mitochondrial membrane and the concomitant loss of the mitochondrial electron carriers (Perez-Pinzon *et al.*, 1999c). In that study, cytosolic cytochrome *c* was increased following global cerebral ischemia and 30 min of reperfusion (same model of cerebral ischemia as in the current study); and conversely, reducible cytochrome *c* (presumably the intramitochondrial fraction of this cytochrome) was decreased following anoxia in hippocampal slices. This latter finding was correlated with NADH hyperoxidation that occurs following anoxia

in hippocampal slices and the first window of preconditioning was unable to protect against such hyperoxidation (Centeno *et al.*, 1999).

IPC and Mitochondrial Protection
in the 2nd Window

In contrast to the lack of mitochondrial protection by IPC in the first window, IPC in the second window significantly protected mitochondria against the deficits in respiration through complexes I–IV (Dave *et al.*, 2001). As described above, many studies have demonstrated that reactive oxygen species (ROS) and the resulting oxidative stress play a pivotal role in neuronal cell death (Flamm *et al.*, 1978; Fridovich, 1979; Hall and Braughler, 1993; Kontos, 1989; Siesjo *et al.*, 1985; Vlessis *et al.*, 1990). There are two major regions in the electron transport chain where ROS are produced. One is complex I and the other complex III (Chance and Williams, 1956). Since oxidative stress is implicated in the pathophysiology that ensues after cerebral and cardiac ischemia (Kannan and Jain, 2000), one can surmise that a key mechanism by which IPC in the second window protects hippocampus against *delayed* neuronal cell death is by protecting mitochondrial oxidative phosphorylation.

However, the precise mechanism by which IPC affords protection to mitochondria remains undefined. A possible mechanism may involve upregulation of neuroprotective genes. Cai and Storey (1996) found that anoxia stress induces upregulation of the genes for NADH-ubiquinone oxidoreductase subunit (encoded by mitochondrial gene) and cytochrome oxidase subunit 1 (encoded by mitochondrial gene) in the anoxic-resistant turtle heart (Cai and Storey, 1996). We presume that IPC may upregulate certain genes responsible for the activities of these complexes, which may render hippocampal mitochondria resistant to "lethal" ischemia.

Another possible mechanism may involve better maintenance of ATP. There is considerable evidence for the role of ATP depletion, which follows hypoxic/ischemic insults, in the development of mitochondrial damage and the subsequent activation of downstream cell death pathways (Galeffi *et al.*, 2000). Yabe *et al.* (1997) have shown that in preconditioned heart, the glycolytic ATP production increases. They have also demonstrated that ATP and creatine phosphate concentration remained higher in preconditioned group as compared to nonpreconditioned group (Galeffi *et al.*, 2000). A higher level of ATP in preconditioned group may prevent cytochrome *c* release from the mitochondria, as was previously observed by Galeffi *et al.* (2000).
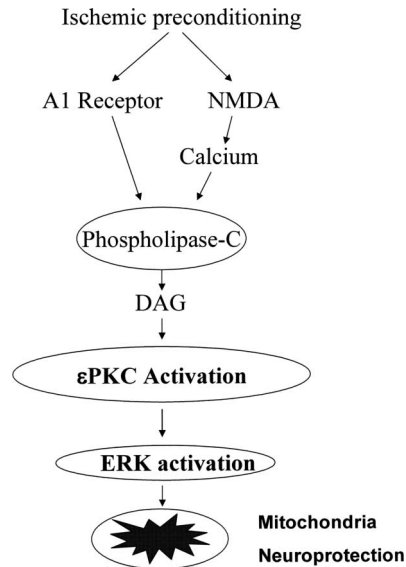
Fig. 2. Flow chart defining the signal transduction pathways leading to ischemic tolerance. We and others have characterized that the adenosine A1 and the NMDA receptors are involved in the triggering phase of IPC. The ensuing pathways lead to εPKC and ERK activation, which we propose may be protecting mitochondria.

Putative Role of the Signal Transduction Pathway on Mitochondrial Protection After Cerebral Ischemia: Role of Protein Kinase C (PKC)

We recently demonstrated that one specific PKC isozyme, namely εPKC, plays a pivotal role in the induction of tolerance after ischemic preconditioning (Lange-Asschenfeldt *et al.*, 2003; Raval *et al.*, 2003; Raval and Perez-Pinzon, 2003) (see Fig. 2). Basu *et al.* (2002) showed that cleavage of εPKC by caspase-7 results in the activation of εPKC, which was associated with its antiapoptotic function. Also, formation of mitochondrial εPKC-ERK$^1/_2$ modules was coupled to the inactivation of BAD, a proapoptotic molecule (Baines *et al.*, 2002). Since, ischemic preconditioning has been shown to preserve mitochondrial function (Dave *et al.*, 2001; Fryer *et al.*, 2000), we hypothesize that εPKC promotes ischemic tolerance by protecting mitochondrial function during the reperfusion phase. However, the precise mechanism by which IPC via εPKC protects mitochondria following cerebral ischemia remains undefined. Further studies from our laboratory are underway to define these mechanisms.

ACKNOWLEDGMENTS

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