Effects of ischemic preconditioning on mitochondrial and metabolic neruoprotection: 5' adenosine monophosphate-activated protein kinase and sirtuins

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Abstract:

Stroke and cardiac arrest result in cerebral ischemia, a highly prevalent medical issue around the world, which is characterized by a reduction or loss of blood flow to the brain. The loss of adequate nutrient supply in the brain during ischemia results in neuronal cell death contributing to cognitive and motor deficits that are usually permanent. Current effective therapies for cerebral ischemia are only applicable after the fact. Thus, the development of preventative therapies of ischemia is imperative. A field of research that continues to show promise in developing therapies for cerebral ischemia is ischemic preconditioning (IPC). IPC is described as exposure to sublethal ischemic events, which induce adaptive changes that provide tolerance to future ischemic events. Through either transient sub-lethal ischemic events, or the actions of a preconditioning molecular mimetic, IPC typically results in augmented gene expression and cellular metabolism. A pivotal target of such changes in gene expression and metabolism is the mitochondrion. Direct and indirect effects on mitochondria by IPC can result in the activation of 5' adenosine monophosphate-activated protein kinase (AMPK), a master regulator of cellular metabolism. Changes in the activity of the posttranslational modifiers, SIRT1 and SIRT5, also contribute to the overall adaptive processes in cellular metabolism and mitochondrial functioning. In this review, we present recently collected evidence to highlight the neuroprotective interactions of mitochondria with AMPK, SIRT1, and SIRT5 in IPC. To produce this review, we utilized PubMed and previous reviews to target and to consolidate the relevant studies and lines of evidence.

Keywords:

5' adenosine monophosphate-activated protein kinase, ischemic preconditioning, metformin, SIRT1, SIRT5

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Introduction

Roughly, every 40 s, an individual in the United States is the victim of a stroke. This life-threatening event will cause periods of cerebral ischemia.[1] Ischemia is defined as the reduction of blood flow to the brain which results in a loss of nutrient and oxygen supply. If ischemia persists, brain damage occurs in the form of excitotoxic and necrotic neuronal death.[2] Therapeutic treatments for stroke are predominantly postischemia, and there has been little success with preventative therapies.[3,4] However, endogenous protective mechanisms have been investigated in the field of ischemic preconditioning (IPC). IPC is defined as a sublethal ischemic insult which induces adaptive changes in cellular function that confer protection against future ischemic events.

Mitochondria and Ischemia

A pivotal target and inducer of IPC in neurons is the mitochondrion.[5] Mitochondria are the major energy-producing constituents of the cell and mitochondrial dysfunction is involved with many deleterious mechanisms in ischemic injury and neuronal cell death. Reactive oxygen species (ROS), which are produced normally by mitochondria, are increased by dysfunctional and idle mitochondria. ROS overproduction results in peroxynitrite formation which can cause inactivation of mitochondrial ATPase, DNA fragmentation, and cell death.[6-8] Ischemia-induced increases in intracellular calcium can result in mitochondrial uncoupling and opening of the mitochondrial permeability transition pore (MTPT). MTPT opening dysregulates ATP production and releases ROS into the cytosol.[9] The mitochondrial apoptosis-induced channel (MAC) is formed by pro-apoptotic Bcl-2 proteins after reperfusion. The MAC releases cytochrome c and apoptosis-inducing factor resulting in reperfusion-mediated neuronal death.[10,11] These mitochondrial cytotoxic mechanisms contribute heavily to ischemic injury.

The major focus of this review will be on the IPC-mediated effects on mitochondrial metabolism and signaling as well as IPC-mediated epigenetic modifications, especially enacted by sirtuins, and how they influence mitochondrial functioning. The synthesis of this review entailed the use of PubMed searches and previous reviews to determine the pertinent studies to incorporate.

5′ Adenosine Monophosphate-Activated Protein Kinase and Metabolic Homeostasis

Role of activated 5′ adenosine monophosphate-activated protein kinase

The 5′ adenosine monophosphate-activated protein kinase (AMPK) is a metabolic sensor within the cell. This protein has three subunits, the catalytic α-subunit and regulatory β, γ-subunits. The γ-subunit has binding sites for the adenosine-phosphate molecules and can sense cellular ATP: ADP and ATP: AMP ratios.[12,13] If ATP is exchanged for AMP at one of the γ-subunit sites, an allosteric change increases AMPK's Thr172 affinity for phosphorylation.[14] Thr172 has been shown to be phosphorylated by upstream kinases; TAK1, CaMKK, or LKB1.[15-17] Combining AMP binding and Thr172 phosphorylation can increase the activity of AMPK by over 1000-fold.[18] When activated AMPK is responsible for adaptive changes in metabolic function which conserves ATP supply.[19]

AMPK activity and mitochondrial dysfunction are linked in instances of ischemia. In ischemic or hypoxic conditions, the oxygen supply within the brain is exhausted within a matter of seconds. This quickly halts mitochondrial ATP production and increases the AMP: ATP ratio, promoting AMPK activation.[20] Common targets of AMPK augment cellular energy production and mitochondrial functioning. Phosphofructokinases can be activated by AMPK to stimulate glycolytic ATP production.[21] The FOXO3a transcription factor can upregulate antioxidant gene expression which improves mitochondrial efficiency.[22,23] Proliferator-activated receptor gamma coactivator 1-α (PGC-1α), a master regulator of mitochondrial biogenesis, is upregulated by FOXO3a.[24] Finally, phosphorylation of TSC2 by AMPK suppresses mTOR and promotes energy producing autophagy.[25] Thus, AMPK-mediated mechanisms respond to energy deficiency by increasing energy production while simultaneously enhancing mitochondrial efficiency and inducing mitochondrial biogenesis. AMPK adapts the metabolic state of the cell soon after ischemia begins, and it has been shown to be a prime regulator for neuroprotective mechanisms of IPC.

5′ Adenosine Monophosphate-Activated Protein Kinase in Ischemic Preconditioning and Neuroprotection

Resveratrol and mitochondrial efficiency

AMPK activation has been shown to be involved in IPC neuroprotection through direct or indirect interactions with mitochondria and through maintaining metabolic homeostasis. IPC mimetics, such as resveratrol and metformin, have been shown to confer neuroprotection through AMPK activation and mitochondrial effects.

Both resveratrol treatment and IPC were shown to activate AMPK through protein kinase C epsilon (PKCε) during preconditioning *in vitro*.[26] PKCε-mediated AMPK activation increased mitochondrial pools of Nampt, the rate-limiting enzyme that drives the formation of NAD. NAD is an integral coenzyme in cellular energy production both in the tricarboxylic acid cycle and electron transport chain.[27] Increased mitochondrial pools of Nampt and NAD were protective against oxygen-glucose deprivation, where AMPK activity was required for this neuroprotection. Both *in vivo* and *in vitro* models of RPC showed neuroprotection through activation of Nrf2, a transcription factor that upregulates antioxidant systems and helps to maintain mitochondrial coupling and antioxidant protein expression.[28] This evidence highlights resveratrol's capability to promote preconditioning by improving mitochondrial efficiency.

Metformin and 5′ Adenosine Monophosphate-Activated Protein Kinase Signaling

Metformin, a common Type II diabetes medication, induces a reduction in glucose production and has been

suggested to be involved in signaling, or induction, of abnormal metabolic conditions.[29] Metformin has recently been implicated as an IPC mimetic. Metformin enacts its pharmacological effects by inhibiting Complex I of the electron transport chain. Metformin's inhibition of Complex I can also promote glycolysis in the cell.[30] Ultimately, metformin reduces ATP production and has been shown to indirectly activate AMPK by increasing the AMP: ATP ratio.[31] Preconditioning with metformin has elicited both neuroprotective and toxic effects, all of which depend on the time-point, duration, and concentration of treatment.[32]

The common understanding is that metformin exerts its effects on ischemic outcomes through AMPK and AMPK-mediated processes.[32] Several *in vivo* studies have shown metformin-AMPK-based neuroprotection. Ashabi *et al.*[33] preconditioned rats with daily metformin treatments for 2 weeks before transient forebrain global ischemia. They found that metformin preconditioning (MPC) increased mitochondrial biogenesis and reduced apoptotic cell death. Factors upstream of mitochondrial biogenesis; PGC-1α, NRF-1, and TFAM, were upregulated in MPC rat hippocampus. Hippocampal CA1 cell death was reduced in MPC rat. If compound c, an AMPK inhibitor, was applied just before ischemia the neuroprotective effects of MPC were lost. This study showed that MPC-activated AMPK can provide neuroprotection through mitochondrial biogenesis and produce anti-apoptotic effects in rat hippocampus. Another *in vivo* study by Ashabi *et al.*[34] investigated AMPK activity in MPC in the rat with a global carotid artery ischemic model. Postischemia inflammatory markers in the hippocampus, nuclear factor-kappa (NF-κB), tumor necrosis factor-alpha and cyclooxygenase-2, were all reduced in MPC animals. Hippocampal Nrf2 levels were increased with MPC. Once again, treatment with compound c before ischemia abolished the downregulation of pro-inflammatory factors and upregulation of Nrf2. Thus, AMPK and its activity, is required for the reduction of inflammation as well as an increase in antioxidant signaling seen with MPC. These effects may increase the efficiency of mitochondrial energy production. Finally, MPC was shown to increase autophagy through its activation of AMPK. MPC rats showed a reduction in infarct volume and apoptotic cell death after permanent middle cerebral artery occlusion while having increased counts of autophagosomes.[35] The same group also found that 10 min of IPC resulted in AMPK-mediated autophagy.[36] AMPK-mediated autophagy may help to increase the ATP supply in ischemic conditions. An overview of AMPK's activation and downstream effects in preconditioning is depicted in Figure 1.

Considering all the evidence discussed it appears metformin-activated AMPK may be an integral component
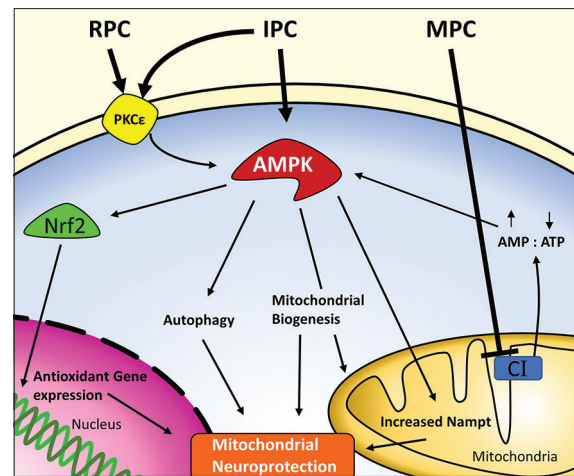

Figure 1: Ischemic preconditioning and its mimetics activate AMPK to induce mitochondrial neuroprotective mechanisms. Ischemic preconditioning, resveratrol, and metformin have all been shown to activate AMPK through various means. Activation of AMPK induces cellular changes in mitochondrial function, mitochondrial number, and antioxidant expression. Ultimately, the activation of AMPK promotes mitochondrial neuroprotection of ischemic insult. AMPK = 5` adenosine monophosphate-activated protein kinase, AMP = Adenosine monophosphate, ATP = Adenosine triphosphate, CI = Complex I, IPC = Ischemic preconditioning, MCP = Metformin preconditioning, Nampt = Nicotinamide phosphoribosyltransferase, Nrf2 = Nuclear factor erythroid 2-related factor, PKCε = Protein kinase C epsilon, RPC = Resveratrol preconditioning

to mitochondrial ischemic neuroprotection in MPC. However, with mixed results, it is imperative that MPC and its activation of AMPK be investigated further.[37]

Epigenetic Changes of Ischemic Preconditioning and Cellular Metabolism

Epigenetic regulation is defined as changes in gene expression without any direct changes to the DNA sequence or code. Epigenetic changes involve the posttranslational modifications of histones, condensing and decondensing of DNA, and the production of noncoding regulatory RNAs that enact regulatory effects on protein-coding mRNAs.[38] There is potential for epigenetic changes to account for the long-term ischemic tolerance afforded by IPC and IPC mimetics.[39] Histone marks and posttranslational modifications are common forms of epigenetic changes thought to confer long and short-term ischemic neuroprotection. Deacetylation and desuccinylation, catalyzed by the Sirtuin proteins, SIRT1 and SIRT5, respectively, have been associated with IPC. SIRT1 is a histone deacetylase, and SIRT5 is a desuccinylase with additional enzymatic functions.[40] Sirtuin activity has been shown to be involved in protection against ischemic injury in IPC through augmentation of mitochondrial function and cellular metabolism.

SIRT1 and Deacetylation

SIRT1 is an NAD⁺-dependent protein deacetylase. SIRT1 catalyzes NAD⁺ into nicotinamide, removing

acetyl groups from lysine amino acid residues. NAD$^+$ is a pivotal metabolic cofactor required for cellular respiration and glycolysis. Therefore, the dependence on the consumption of NAD$^+$ links SIRT1 as a metabolic sensor. SIRT1's expressional regulation is sensitive to the metabolic state of the cell through the availability of NAD$^+$. This regulation can be achieved by SIRT1 histone deacetylation, resulting in transcriptional depression; or by the interaction of SIRT1 with other nuclear enzymes, either leading to a positive or negative transcriptional modulation of target genes.[2,41]

SIRT1 enzymatic activity is increased after IPC or RPC in an *ex vivo* model, the rodent organotypic hippocampal slice.[41] Consistent with *ex vivo* results, SIRT1 activity is increased *in vivo* in rat hippocampus following IPC or RPC.[42] Aside from SIRT1 enzymatic activity, brain SIRT1 protein levels are decreased in rats exposed to MCAO, linking protein levels to the oxygen state of the cell. Hyperbaric oxygen (HBO) preconditioning enhances SIRT1 expression in the rat brain and protects against MCAO.[43] Furthermore, SIRT1 and its activity has been shown to be required for IPC. Inhibition of SIRT1 with sirtinol abolishes IPC or resveratrol-mediated neuroprotection against OGD in organotypic hippocampal slices. In rodent models, neuronally specific mutated SIRT1 abrogates RPC neuroprotection in a mouse MCAO model.[44] Similarly, HBO-induced neuroprotection against MCAO is lost after SIRT1 knockdown by shRNA in the rat brain.[45] It is evident that SIRT1 and its activity is required for numerous IPC and RPC models, suggesting that SIRT1 is an essential regulator of ischemic neuroprotection.

SIRT1 has been shown to mediate its neuroprotection against cerebral ischemia through various mechanisms. As a potent SIRT1 activator, resveratrol increases Nrf2 protein expression, which upregulates mitochondrial antioxidant availability and protects against MCAO in mouse.[28] Other evidence shows that, in the rat brain, HBO preconditioning increases Nrf2 and SIRT1 expression levels. SIRT1 knockdown decreases Nrf2 expression while Nrf2 knockdown does not affect the expression of SIRT1.[45] This directly supports Nrf2 as a downstream effector in the SIRT1 signaling pathway. In addition, SIRT1 has been shown to protect against ischemic injury through its deacetylase activity. SIRT1 inhibition has been shown to increase acetylation of p65, which results in the activation of the pro-inflammatory transcriptional factor NF-κB.[46] Ischemia-induced hyperacetylation of SIRT1 downstream effectors has been shown to cause mitochondrial damage and neuronal cell death.[47] In the rat brain, HBO preconditioned MCAO-rats have decreased levels of acetylated p53 and NF-κB in comparison to the MCAO-only group, while this decrease is abolished by the SIRT1 knockdown. This suggests

HBO-induced neuroprotection is dependent on SIRT1 deacetylase activity.[45] In addition, resveratrol-induced SIRT1 activity has been shown to decrease the expression of mitochondrial uncoupling protein two (UCP2). In rat hippocampus mitochondria, UCP2 protein levels were downregulated 48 h after resveratrol treatment. As a result, the ratio of oxygen consumption to ATP produced, was enhanced, suggesting an increase in mitochondrial ATP synthetase efficiency.[42] Recently, it has been reported that SIRT1 plays a role in glycolysis regulation, another important aspect of ATP synthesis.[44] Abnormal glucose metabolism in the neuronal SIRT1 mutant mouse brain was detected through metabolic analysis. Further evidence showed glycolytic ATP production was increased after RPC *in vitro*. This increase was impaired by pharmacological inhibition of SIRT1. Similarly, glycolysis is impaired in the acute brain slices from neuronal SIRT1 mutant mouse compared to wild type.[44] Overall, Sirt1 confers its mitochondrial neuroprotection against ischemic injury through diverse mechanisms. Further studies of the clinical application of SIRT1 modulators would be of great interest.

SIRT5 Regulates Several Metabolic Processes during Ischemia

SIRT5, a sirtuin known for its role in regulating the urea cycle in the liver, has recently become a target of interest in ischemia given its potential impact on mitochondrial metabolism. Under conditions when glucose is abundant, cells overexpressing SIRT5 can increase glycolysis, mitochondrial substrate oxidation, oxygen consumption, and mitochondrial proton leak.[48,49] Although SIRT5 is mainly localized in the mitochondrion, levels have also been detected in the cytosol and nucleus, which may explain one aspect of its functional diversity.[48,50,51]

As with all other sirtuins, SIRT5 possesses intrinsic, albeit weak, deacetylase activity. In addition, SIRT5 can remove other acyl groups such as malonyl, succinyl, and glutaryl on lysine residues.[52] Studies have indicated that SIRT5 prefers these substrates over acetyl-lysine.[49] Notably, SIRT5 is the only histone deacetylase to catalyze lysine desuccinylation and demalonylation.[53]

In the heart, SIRT5 functions as a regulator of fatty acid metabolism, oxidative phosphorylation, and other metabolic pathways.[54-56] SIRT5 knockout (SIRT5 KO) mice were shown to be more susceptible to ischemia/reperfusion (I/R) injury, mainly due to a heightened rate of succinate oxidation catalyzed by succinate dehydrogenase (SDH) during reperfusion.[54] Succinate has been found to increase substantially in the heart, brain, and other tissues following ischemia.[57] The rapid oxidization of succinate drives the production of ROS that initiates I/R injury in the heart.[54,57] In both

SIRT5 KO mice and *Sirt5* knockdown mouse embryonic fibroblasts (MEFs), the lack or reduction of lysine desuccinylation resulted in increased SDH succinylation and activity.[50,54] In both cases, SIRT5 functioned as a suppressor of SDH activity that was coupled with a reduction in cellular respiration.

Sirt5 deficient MEFs showed higher ROS levels, suggesting a protective role in oxidative stress for SIRT5.[56] This was due to a decrease in the production of NADPH, an essential cofactor involved in maintaining glutathione in its reduced form (GSH). GSH acts as an antioxidant, ROS scavenger, and when reduced results in ROS accumulation.

However, a potential neuroprotective role for SIRT5 in the brain is less clear. It has been previously shown that ischemic neuroprotection afforded by the specific PKCε activator, ΨεRACK, requires SIRT5 activity *in vitro* and *in vivo*.[58] ΨεRACK treated wild-type mice subjected to 85 min of MCAO displayed a reduction in infarct volume 48 h postfocal ischemia induction, whereas SIRT5 KO mice did not. In addition, both levels of mitochondrial respiration and lysine desuccinylation activity were increased on ΨεRACK treatment.[51,58] This is contrary to what was observed in heart and MEFs, where cellular respiration was decreased in the presence of SIRT5. SIRT5 may contribute to the differences in mitochondrial respiration between tissues through its regulation of several mitochondrial metabolic proteins through lysine desuccinylation.[50,59,60] Metabolomics studies have also indicated that SIRT5 can regulate central metabolic pathways in the brain that link its activity to ischemic tolerance.[51] These findings suggest a neuroprotective role for SIRT5 in cerebral ischemia.

However, a recent study found a reduction in infarct volume and an improvement in neurological deficits 48 h after transient MCAO in SIRT5 KO mice.[61] They demonstrated that SIRT5 elicited these harmful effects by increasing blood-brain barrier permeability and disrupting tight junction proteins.[61] These findings suggest SIRT5 may mediate brain injury.

Discrepancies in these outcomes may be explained by differences in focal ischemia induction. The latter study subjected mice to 45 min of MCAO, whereas the former used longer durations (85 min).[58,61] One study found that reactive astrogliosis was enhanced in mice subjected to 45 min of ischemia, accompanied by only selective neuronal loss, while mice who underwent ≥60 min of MCAO endured a massive loss of both neurons and astrocytes.[62] Interestingly, ciliary neurotrophic factor-induced astrogliosis was found to increase fatty acid β-oxidation—a catabolic pathway that is also enhanced by SIRT5 activity.[55,63] ATP generation through β-oxidation increases the risk of neurons becoming hypoxic and exacerbates oxidative stress in the brain.[64] Perhaps during shorter periods of MCAO, SIRT5 activity may increase β-oxidation in astrocytes resulting in the generation of ROS, leading to more damage and greater infarct size. This may explain the observed reduction in infarct volume in SIRT5 KO mice. It may be that longer durations of MCAO attenuate reactive astrogliosis and β-oxidation as more astrocytes begin to die. At this time, SIRT5 may exert different effects on energy metabolism that are protective rather than detrimental. More work needs to be done to elucidate the exact role of SIRT5 concerning energy metabolism and its potential impact on the brain following ischemia. Cell type-specific studies of SIRT5 activity in the brain will become integral to determine the specific SIRT5 mediated changes in metabolism.

SIRT5's Potential for Ischemic Tolerance

SIRT5 is involved in mitochondrial function in instances of ischemia although evidence has been limited and in some areas, conflicting. Many studies have shown that the loss of SIRT5 can exacerbate ischemic injury, suggesting SIRT5 may play a protective role.[54,56,57] In addition, SIRT5 has been shown to be involved in some forms of IPC. SIRT5 is required for the neuroprotection afforded by PKCε preconditioning in the mouse.[58] SIRT5 may mediate this preconditioning activity by promoting a more neuroprotective metabolic profile.[51] In this way, SIRT5 may be able to adapt components of mitochondrial metabolism to be more tolerant to ischemic conditions. SIRT5 studies have produced complicated results; however, the role of SIRT5 in IPC is understudied and warrants further investigation. A greater understanding of SIRT5 may provide insights into unique mitochondrial mechanisms of ischemic tolerance.

Interactions of 5' Adenosine Monophosphate-Activated Protein Kinase and SIRT1

AMPK, SIRT1, and SIRT5 are all sensitive to aspects of cellular metabolism. Portions of the activities of these metabolic sensors regulate mitochondrial function. There is evidence that SIRT1 and AMPK interact; however, little is known about potential interactions between these proteins and SIRT5. The activity of SIRT1 and AMPK converge on the factor PGC-1α to induce mitochondrial biogenesis. SIRT1's sensitivity to the NAD^+: NADH ratio, and AMPK's sensitivity to the AMP: ATP ratio, links these two metabolic balances in their promotion of mitochondrial biogenesis.[65] In a rat intestinal I/R model conducted by Jing *et al.*, postischemic rats were treated with fish oil, which attenuated ischemic injury through the AMPK-SIRT1-autophagy pathway.[66]

Resveratrol-induced SIRT1 activation has been shown to result in AMPK activity.[67] Resveratrol-mediated SIRT1 and AMPK cooperative activity is seen in autophagy in a Parkinson's Disease model, the regulation of glucose and lipid homeostasis in the kidney, and even a reduction of protein translation to reduce tumor cell proliferation.[67-69] With a known IPC mimetic, such as resveratrol, resulting in both SIRT1 and AMPK activity, there is a need to investigate potential mechanisms between these factors in contexts of cerebral ischemia and preconditioning. In addition, the mitochondrial SIRT5's potential role in IPC deserves further investigation to truly understand all the effects of IPC on the mitochondrion.

Conclusion

Cerebral ischemia and the damage it creates is a complicated and multifaceted problem that requires further investigation to elucidate effective preventative therapies. Currently, IPC and IPC mimetics provide the research community with windows to observe and to learn from endogenous adaptive mechanisms in the face of ischemia. Although not extensively covered in this review, new insights may be obtained from cardioprotection and other tissue types when it comes to IPC that could potentially be translatable to cerebral ischemia. The mitochondrion has, and will continue to be, a pivotal target for enhancing the cells' ability to withstand ischemic events. Thus, it is important to continue to investigate the effects of global metabolic regulators such as AMPK and epigenetic and posttranslational modifiers such as SIRT1 and SIRT5. The more we understand the underlying mechanisms utilized by these factors to augment the efficiency and function of mitochondria in IPC the more we will be able to experimentally, and eventually clinically, manipulate the metabolic homeostasis of neurons to confer protection from ischemic insult.

Conflicts of interest
There are no conflicts of interest.

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