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Preconditioning and tolerance against cerebral ischaemia:

from experimental strategies to clinical use

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Abstract

Neuroprotection and brain repair in patients after acute brain damage are still major unfulfilled medical needs. Pharmacological treatments are either ineffective or confounded by adverse effects. Consequently, endogenous mechanisms by which the brain protects itself against noxious stimuli and recovers from damage are being studied. Research on preconditioning, also known as induced tolerance, over the past decade has resulted in various promising strategies for the treatment of patients with acute brain injury. Several of these strategies are being tested in randomised clinical trials. Additionally, research into preconditioning has led to the idea of prophylactically inducing protection in patients such as those undergoing brain surgery and those with transient ischaemic attack or subarachnoid haemorrhage who are at high risk of brain injury in the near future. In this Review, we focus on the clinical issues relating to preconditioning and tolerance in the brain; specifically, we discuss the clinical situations that might benefit from such procedures. We also discuss whether preconditioning and tolerance occur naturally in the brain and assess the most promising candidate strategies that are being investigated.

Introduction

Organisms have evolved mechanisms to protect against tissue damage and to compensate (or even regenerate) in the event of injury. The two most elementary challenges, and thus the greatest evolutionary pressures, for living organisms are infection and deprivation of substrate or energy. Pathophysiological research has focused on mechanisms by which tissue is damaged by noxious stimuli or processes and how to prevent this injury.

To identify endogenous mechanisms of protection and repair, and to make use of these mechanisms therapeutically, biomedical investigators have developed preconditioning strategies. Preconditioning is a procedure by which a noxious stimulus near to but below the threshold of damage is applied to the tissue. Shortly after preconditioning or after a delay, the organ (and therefore the organism) develops resistance to, or tolerance of, the same, similar,

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Conflicts of interest

AM and UD are the patent inventors of a patent application on a neuroprotective compound (an erythropoeitin derivative) that has been filed to the European Patent Office (PCT/EP2006/004564). Charite Universitätsmedizin Berlin is the patent owner. KB is the patent inventor of US patent 08/994,293, which focuses on the use of mucosal tolerance for the treatment of stroke. The US Department of Health is the patent owner.

or even different noxious stimuli given beyond the threshold of damage. Preconditioning thereby protects against subsequent injury.

Ischaemic brain injuries, resulting either from global or focal decreases in perfusion, are among the most common and important causes of disability and death worldwide. The consequences of global cerebral ischaemia after cardiac arrest (and successful resuscitation), focal occlusions or disruption of brain vessels (ie, stroke, including subarachnoid haemorrhage and intraparenchymatous haemorrhage), and ischaemic brain damage after cardiac or brain surgery affect many millions of people in the USA alone.[1,2] Research into preconditioning aims at developing new therapeutic approaches to benefit these patients. On the one hand, preconditioning is an attractive experimental strategy to identify endogenous protective or regenerative mechanisms that can be therapeutically induced or supplemented. On the other hand, preconditioning could be used as a therapeutic technique by inducing tolerance in individuals in whom ischaemic events are anticipated, such as high-risk surgical cohorts or patients with subarachnoid haemorrhage or transient ischaemic attack. Many articles have reviewed various features of ischaemic preconditioning, tolerance, and endogenous neuroprotection in the brain.[3-18] In this Review, we give a brief overview of preconditioning, including ischaemic preconditioning, and its clinical potential and discuss the therapeutic exploitation of endogenous neuroprotection. Additionally, we hope to expand the neurocentric view of preconditioning and tolerance held by neuroscientists and neurologists to include the immune system.

Induction of ischaemic tolerance

Many pathological pathways converge on shared pathways of cell injury, death, and repair. For example, although the causes of acute neurodegeneration (eg, stroke) and chronic neurodegeneration (eg, Parkinson's disease) are different, the mechanisms of cell injury—including excitotoxicity, inflammation, and apoptosis—overlap,[19] as do the pathways of survival and regeneration. Therefore, the options for inducing preconditioning and tolerance are not specific to the type of injury, which is important for the clinical adaptation of this technique. The table gives an overview of the different types of preconditioning (cross, remote, immunological, pharmacological, anaesthetic, mimetic, and effector).[20-47]

Preconditioning can protect the brain either almost immediately after stimulation (known as early, rapid, or classical preconditioning) or after a delay of 1 to 3 days to induce protein-synthesis-dependent protection (delayed preconditioning). Most stimuli can cause both early and delayed preconditioning, and most, but not all, stimuli leave an unprotected time window between early and delayed preconditioning.[24] Irrespective of the rapidity of onset, protection by preconditioning usually never lasts more than a few days. Of note, a recent study showed that a series of repetitive hypoxic preconditioning stimuli can induce neuroprotection in the retina that last many weeks. Such long-term tolerance might be associated with neuronal plasticity, including long-term potentiation, or long-lasting cellular memory associated with immune tolerance.[48]

Rapid preconditioning is appealing practically and clinically because this technique can be applied therapeutically in the same setting as procedures with high risks of complication, such as cardiac or brain surgery. Most of the experimental and clinical research in cardiology has thus focused on early preconditioning. Conversely, because protection conferred by delayed preconditioning seems to be more robust for the brain than that conferred early, delayed preconditioning has received more attention in neurology. However, although there are effective protocols for early preconditioning for the brain,[49] there are few formal comparisons of early and delayed procedures for neuroprotection.[50]

The recently described event of postconditioning is commonly discussed in the context of preconditioning. Postconditioning is a form of therapeutic reperfusion by which an organ is intermittently reperfused, for example by a stuttering opening and closing of an experimentally (or clinically, as in angioplasty or in organ transplantation) occluded artery. Postconditioning is a new neuroprotective approach for lessening injury in focal ischaemia and reperfusion.[51] The benefits of this procedure seem to be mediated, at least in part, by molecular pathways similar to those that control preconditioning.[52] As an experimental strategy postconditioning, like preconditioning, might lead to the discovery of endogenous mechanisms of protection and repair. However, postconditioning is unlikely to be of clinical relevance as a therapeutic strategy for ischaemic brain damage, and therefore will not be discussed further in this Review.

Genomic reprogramming

The induction of ischaemic tolerance is accompanied by substantial change in gene expression, suggesting that preconditioning stimulates a fundamental genomic reprogramming of cells that confers cytoprotection and survival.[6] The genomic response after ischaemic preconditioning is a signature of the complex interplay of multiple signalling pathways. These highly specialised pathways in different cell types of the brain seem to refine the cellular and systemic response to combat the noxious stimulus. Hundreds of genes are either upregulated or downregulated in response to ischaemic preconditioning stimuli.[53-55] Changes in gene expression differ between harmful ischaemia and ischaemic preconditioning. Preconditioning seems to attenuate the response to ischaemia.[54] Tolerance induced by ischaemic preconditioning changes the expression of genes involved in the suppression of metabolic pathways, immune responses, ion-channel activity, and blood coagulation.[54]

Gene expression is regulated by transcription factors but also depends on epigenetic mechanisms such as DNA methylation and histone modification, which modify the chromatin structure that controls access of transcription factors to regulatory loci. Inhibition of DNA methylation and increased histone acetylation have neuroprotective effects in experimental models of stroke.[56] Ischaemic preconditioning induces substantial changes in acetylation of the H3 and H4 histones, which are associated with neuroprotection (Yildrim F, Meisel A, unpublished). These changes seem to facilitate widespread changes in transcription and support the concept of genomic reprogramming by preconditioning. Pharmacological inhibition of histone deacteylases with trichostatin A leads to increased histone acetylation and has a neuroprotective effect.[56-62] Inhibition of histone deacteylases increases the production and function of regulatory T cells[63] and might thereby augment protective immune mechanisms associated with endogenous tolerance. Epigenetic mechanisms of gene regulation might therefore provide another avenue of therapeutic neuroprotection.[62]

Hypoxia-inducible factor: a regulator of ischaemic preconditioning?

One of the key regulators of the genomic response after ischaemic preconditioning is the transcriptional activator hypoxia-inducible factor (HIF). This protein is a heterodimer with an unstable α-subunit (HIFα) and a stable β-subunit (HIFβ).[64] HIF is regulated by an evolutionarily conserved pathway mediated by oxygen-dependent post-translational hydroxylation of HIFα. Under typical oxygen conditions, HIFα becomes hydroxylated at two prolyl residues by members of the prolyl-4 hydroxylase domain family, generating a binding site for a component of the ubiquitin ligase complex. Polyubiquitination tags HIFα for proteasomal degradation. The activity of prolyl-4 hydroxylase domain proteins is fully dependent on oxygen. Under hypoxic conditions, HIFα is not degraded; it accumulates, dimerises with the HIFβ subunit, and transactivates about 100 genes. These genes encode proteins involved in oxygen transport (erythropoietin), angiogenesis (vascular endothelial growth factor [VEGF] and angiopoietin-2), vasomotor control (adrenomedullin and β-

adrenergic receptors), cell survival (VEGF and erythropoietin), pH regulation (carbonic anhydrases), and energy metabolism (glucose transporters and glycolytic enzymes).[64] HIF might thereby support tissue oxygenation and cellular energy preservation after cerebral ischaemia. Ischaemic preconditioning activates HIF1—the best known member of the HIF family—and its target genes.[65-71] HIF1 activation is neuroprotective,[47] whereas a neuron-specific HIF1α deletion exacerbates brain injury in an experimental model of stroke.[72] Furthermore, HIF and its target gene *VEGF* are upregulated in neurons that are connected to, but remote from, the infarcted area.[73] Because neurons projecting to an infarcted lesion are at risk of delayed neuronal death through loss of trophic support (eg, growth factors and excitation) by surrounding cells in the damaged area, upregulation of trophic cytokines might prevent neuronal death and support axonal sprouting into the penumbra.[74]

By contrast with the events in focal cerebral ischaemia,[72] neuron-specific HIF1α deletion decreased brain damage in a mouse model of global ischaemia.[75] Proapoptotic genes are downregulated, whereas prototypical HIF-dependent neuroprotective genes are regulated similarly in the absence and presence of HIF1α in the brain, suggesting a functional redundancy of their transcriptional control in this experimental strategy.[75] This suggestion is supported by the observation that the transcriptional response to hypoxia is controlled not only by HIF1α but also by EPAS1 (endothelial PAS domain protein 1; formerly known as HIF2α).[76,77] Additionally, HIF1α knockdown mice maintain their ability to develop ischaemic tolerance as a result of ischaemic preconditioning,[72] supporting the conclusion that HIF1α is not essential for conferring robust neuroprotection. However, the situation is much more complex because HIF transactivates not only prosurvival factors but also proapoptotic proteins such as BNIP3.[78,79] As with other regulating factors involved in the stress response, HIF coordinates both cell survival and death mechanisms.[47]

HIF1 indisputably has an important role in the complex transcriptional response to hypoxic-ischaemic brain damage; however, whether the effects of HIF1 activation are predominantly beneficial for the CNS is still unclear.[80] Therefore, a comprehensive understanding of the prosurvival and pro-death mechanisms of HIF is crucial for developing new pharmacological strategies for stroke treatment. The prolyl-4 hydroxylase domain proteins, which regulate HIF, are promising targets.

Of the three isoforms, prolyl-4 hydroxylase domain 2 is most important for HIF regulation;[81] the neuroprotective effect of inhibitors of these proteins is dependent on the ability of those inhibitors to activate HIF1.[72] Prolyl-4 hydroxylases belong to the iron (II) and 2-oxoglutarate-dependent family of oxygenases.[82] Chelators of iron such as desferrioxamine induce not only HIF1 and its target genes but also neuroprotection.[39,66,83] Because iron chelators might have many unwanted side-effects, more specific HIF-stabilising prolyl-4 hydroxylase domain antagonists that compete with the binding of the natural cofactors might provide neuroprotection in stroke.[47,84] Some of these small-molecule inhibitors such as FG-2216 are being investigated in phase II clinical trials in anaemic patients with chronic kidney disease.

Improving outcome after stroke

The endogenous response aimed at improving outcome after decreased substrate delivery to the brain (ie, ischaemia), which is at least partly mediated by HIF, relies on four basic actions: increased substrate delivery, decreased energy use, antagonised mechanisms of damage, and improved recovery. Preconditioning can modulate all four of these actions.

Increased substrate delivery

Substrate delivery to the brain depends mainly on cerebral perfusion. At the cellular level, delivery is mainly affected by substrate storage and uptake. Perfusion changes in cerebral blood

flow might be implicated in ischaemic tolerance. Many studies investigating cerebral perfusion in animals that did and did not receive preconditioning in the acute phase after detrimental cerebral ischaemia showed no notable increases in cerebral blood flow in the tolerant state.[85-88] By contrast with the acute phase, an increase in cerebral blood flow in the subacute phase after ischaemia is seen in the penumbra of cerebral ischaemic lesions in animals that have received preconditioning.[89] Furthermore, ischaemic tolerance is associated with the preservation of microvascular perfusion during stroke.[90] In immature brains, preconditioning attenuates the decrease in cerebral blood flow during severe ischaemia. The preservation of cerebral blood flow is accompanied by the induction of many genes involved in vasoregulation and angiogenesis, as well as by an increased vascular density as early as 24 h after preconditioning.[91] Angiogenesis has an important role in neuroregeneration after stroke[67,92] and the growth of new vessels is stimulated by the VEGF and erythropoietin cytokines that are regulated by HIF1.[67,93,94] Adrenomedullin, another HIF1-regulated cytokine, is also involved in ischaemic tolerance.[95] This cytokine improves recovery after stroke and is associated with an induction of angiogenesis and increased cerebral blood flow.[96,97] Ischaemic preconditioning enhances angiogenesis within the first 2 weeks of stroke in the ischaemic penumbra.[98]

The decrease in post-ischaemic brain oedema by ischaemic preconditioning has been attributed, at least in part, to the protective effects of preconditioning on cerebral endothelial cells.[99] Post-ischaemic microvascular endothelial apoptosis is mitigated by ischaemic preconditioning via the induction of the phosphoinositide 3 (PI3)-Akt kinase pathway.[100] The kinase activates survivin, which antagonises apoptosis-inducing factor.[100-102] The PI3-Akt kinase pathway is crucial for inducing the tolerant state in the CNS[103,104] and is mainly activated by growth factors and cytokines (such as VEGF and erythropoietin), which induce antiapoptotic mechanisms. Nitric oxide generated by endothelial nitric oxide synthase is important for vascular function, and nitric oxide has vasodilatory, anti-inflammatory, and antithrombotic properties. Augmentation of nitric oxide production increases cerebral blood flow, which can lead to neuroprotection during brain ischaemia. Endothelial nitric oxide synthase regulated by the PI3-Akt kinase signalling cascade contributes to ischaemic tolerance.[105]

Astrocytic glycogen, which provides the main energy reserve in the brain during cerebral ischaemia,[106] is neuroprotective.[107,108] Preconditioning in immature brains increases glycogen and delays energy depletion caused by ischaemia,[109] which suggests that glycogen contributes to energy preservation during cerebral ischaemia. Glucose transport is crucial for neuronal survival during anoxia. Preconditioning increases glucose transport activity, whereas blocking glucose uptake in neurons abrogates anoxic tolerance induced by preconditioning.[110]

Metabolic downregulation?

Metabolic downregulation, as well as channel arrest, are major mechanisms of hibernation and anoxic tolerance in vertebrate species.[111] Preconditioning induces evolutionarily conserved responses to low blood flow and oxygen availability that also occur during hibernation.[6,104,112,113] Preconditioning regulates genes that decrease ATP use or lead to channel arrest and thereby might decrease energy metabolism.[54,114] However, direct measurement of brain energy metabolism has not shown a clear metabolic downregulation after preconditioning.[114]

Ischaemic preconditioning preserves mitochondrial membrane integrity and function after severe ischaemia.[115-117] Several mechanisms that refine mitochondrial function have been described.[3,11] Tolerance, at least in part, is based on HIF1-dependent reprogramming of the basal cellular metabolism that involves mitochondrial genes.[118,119] HIF might actively downregulate oxidative metabolism by lowering mitochondrial biogenesis, augmenting

glycolysis, decreasing metabolite entry into the citric acid cycle, promoting removal of free-radical-generating mitochondria by autophagy,[120,121] and optimising respiratory efficiency by the differential regulation of cytochrome oxidase subunits.[119]

Antagonism of mechanisms of damage

Neuronal excitotoxicity is important in the pathophysiology of cerebral ischaemia. Excessive extracellular concentrations of the excitatory neurotransmitter glutamate are neurotoxic.[122] Ischaemic preconditioning might induce tolerance either by lowering excessive glutamate release or by increasing glutamate uptake. This preconditioning inhibits excitatory pathways through the downregulation of NMDA and AMPA receptors[123,124] and ameliorates excitotoxicity.[125] Additionally, a shift from excitatory glutamate-mediated neurotransmission to inhibitory GABA-mediated neurotransmission was seen in the tolerant state.[114,125] Glutamate uptake by specific transporters is the most effective mechanism to maintain glutamate concentrations below excitotoxic concentrations.[126] The glial glutamate transporter 1-excitatory amino acid transporter 2 (GLT1-EAAT2) is involved in the bulk of glutamate transport in the brain,[127] and specific overexpression of the GLT1-EAAT2 transporter in astrocytes is neuroprotective.[128] β-lactam antibiotics induce neuroprotection via upregulation of the glial GLT1-EAAT2 glutamate transporter. Ischaemic preconditioning causes upregulation of this transporter in astrocytes via the ligand-activated transcription factor peroxisome proliferator-activated receptor γ (PPARγ), leading to a subsequent decrease in ischaemia-induced glutamate release[129,130] and brain ischaemic tolerance.[131] In human beings, increased plasma concentrations of the endogenous PPARγ receptor agonist 15d-prostaglandin J2 are associated with better outcome in ischaemic stroke.[132] Agonists of the PPARγ receptor are also neuroprotective in experimental models of stroke.[133,134]

The neuronal cellular environment—including astrocytes, microglia, and endothelium—protects neurons against ischaemia. Several cytokines that act in an autocrine and paracrine manner are involved in endogenous neuroprotection. For example, preconditioning stimulates the expression of several HIF1-dependent cytokines (eg, erythropoietin, VEGF, and adrenomedullin). Neuroprotection induced by preconditioning is mediated partly by these cytokines,[70,71,95,135] which exert neuroprotective effects via the PI3-Akt kinase pathway, thereby blocking proapoptotic mechanisms either by upregulation or activation of antiapoptotic proteins such as BCL2L1 (formerly known as BCLXL), BCL2, and survivin or by inactivation of proapoptotic regulators such as BAD.[70,117]

Improved recovery

Ischaemic preconditioning induces the expression of gene programmes involved not only in cytoprotection but also in restorative mechanisms such as neurogenesis and angiogenesis. In adult life, neurons are continuously generated from the progenitor cells in the subventricular zone of the lateral ventricles and the subgranular zone in the hippocampal dentate gyrus. Neurogenesis can be triggered by tissue injury, and ischaemic stroke enhances endogenous neural progenitor cell proliferation and differentiation.[136-140] Although most studies found inflammation and microglia activation detrimental to the survival of the new neurons, recent experimental evidence indicates that, under certain circumstances, microglia can be beneficial and can support neurogenesis, progenitor proliferation, survival, migration, and differentiation.[141] Strategies to increase endogenous neurogenesis might be desirable to improve recovery.[142]

Ischaemic preconditioning encourages cell survival and differentiation of neural progenitor cells.[98,143,144] Proliferation and differentiation of progenitor cells is mainly controlled by growth factors.[93,136,145] Ischaemic preconditioning stimulates the expression of many growth factors, including insulin-like growth factor 1, fibroblast growth factor 2, transforming

growth factor β1, epidermal growth factor, brain-derived neurotrophic factor, erythropoietin, VEGF, glial-derived growth factor, and platelet-derived growth factor A.[69-71,93,135,143,146-149] Such growth factors are involved in ischaemic tolerance and improve outcome in experimental stroke.[135,150-157] Although these growth factors have antiapoptotic and anti-inflammatory effects, part of their effect might be attributable to their role as mediators of a neurorestorative response. The presence of newly generated glial and neuronal precursors might be a neurorestorative response to ischaemic preconditioning.[143,158] The proposed mechanisms for endogenous neurogenesis include neuronal replacement, self-regeneration, and release of neurotrophic substances.[159] Angiogenesis, which is augmented by ischaemic preconditioning[98] and stimulated by growth factors,[93,155] plays an important part in neuroregeneration after stroke.[92] Proliferating endogenous progenitor cells seem to have not only neurorestorative properties but also direct neuro-protective effects that are essential to the establishment of tolerance after preconditioning.[160] The discovery of endogenous regenerative capacity in the mature CNS will improve our understanding of how brains can heal themselves and might provide new therapeutic strategies.[161]

Immunological tolerance

Activation of the innate immune response is a consequence of stroke, and preclinical data indicate that inflammation in the immediate post-stroke period contributes to ischaemic brain injury.[162] Clinical data attribute a detrimental role to post-stroke inflammation. The most convincing of these data came from a trial aimed at preventing leucocyte influx into ischaemic brain tissue. The protein used in this trial, however, induced a systemic inflammatory response associated with worse outcome.[163] Furthermore, patients who develop infection after stroke have worse stroke outcome; this association, in part, indicates that patients with more severe strokes are at high risk of infection, but also suggests that the systemic inflammatory response accompanying an infection has a detrimental effect on the ischaemic brain.[164]

Inflammatory stimuli can also induce ischaemic tolerance. For example, preconditioning with lipopolysaccharide, a potent trigger of the innate immune response, stimulates anti-inflammatory and suppresses proinflammatory pathways.[6] The production of proinflammatory cytokines is initiated by signalling cascades involving Toll-like receptors, which recognise host-derived molecules released from damaged tissue as well as common molecular motifs of invading pathogens. These proteins can exacerbate ischaemic injury;[165-167] for example, a polymorphism in the gene that encodes Toll-like receptor 4 is associated with ischaemic stroke.[168] Receptors 2, 4, and 9 are mediators of ischaemic tolerance in the brain,[7,169,170] and others might be involved.[9] Ligands of Toll-like receptors, such as lipopolysaccharide, induce a state of tolerance to subsequent ischaemia.[171] Although recent data indicate that stimulation of Toll-like receptors leads to specific genomic reprogramming that is different from the gene expression pattern induced by ischaemic preconditioning, the degree of protection is similar. Toll-like receptors are thus promising targets for neuroprotection.[7,171] Small doses of lipopolysaccharide or other agonists of these receptors given before an ischaemic insult can be protective, whereas the inflammatory response associated with infection after stroke can be detrimental. The timing and dose of stimulus application are therefore important to mediate the effects of preconditioning.

Cerebral ischaemia might activate adaptive immunity and innate immunity. The adaptive immune response is characterised by lymphocytes that recognise and respond to specific antigens; however, this response depends on the environment in which the lymphocyte was initially familiarised to the antigen.[172] For a lymphocyte to become activated to an antigen, that antigen must have been encountered in the context of the MHC II and a co-stimulatory signal must be received. The cytokine milieu at the time of encounter then drives the differentiation into different effector cell types. For example, T-helper-1 cells, which are

important for cell-mediated immune reactions, develop in the presence of interferon γ, and T-helper-2 cells, which are important for humoral immunity and the response to parasitic infection, develop in the presence of interleukin 4.[173] T-helper-17 cells are newly described CD4-positive effector cells associated with autoimmune disease. These cells develop in the presence of interleukin 6 and transforming growth factor β1. If the cell encounters its antigen in the presence of transforming growth factor β1 but not interleukin 6, a regulatory T cell develops. A separate type of regulatory T cell develops in the presence of interleukin 10. These inducible regulatory T cells secrete cytokines that modulate the immune response (either transforming growth factor β1 or interleukin 10) and are distinct from naturally present regulatory T cells, which function through direct cell contact.[173]

Because inflammation seems to worsen stroke outcome, induction of regulatory T cells to limit CNS inflammation presents a new opportunity for stroke treatment. Such regulatory T cells can be induced with mucosal tolerance, which can be described as immunological preconditioning when done before an ischaemic insult. Although these cells are activated in an antigen-specific way, they secrete cytokines that modulate the immune response in an antigen non-specific way. Inducible regulatory T cells can thus be used to control the immune response wherever their cognate antigen is present, irrespective of whether that antigen brings about the immune response, an event referred to as bystander suppression.[174] In experimental models of stroke, regulatory T cells primed to the CNS antigens myelin basic protein and myelin oligodendrocyte glycoprotein were associated with decreased infarct size for both antigens.[175-177] Animals with an increased number of regulatory T cells specific to myelin basic protein also have improved long-term outcome as assessed by several behavioural measures.[178] Induction of regulatory T cells to the vascular antigen E-selectin is associated with similar benefits, decreasing the risk of stroke in spontaneously hypertensive stroke-prone rats and reducing infarct volume.[179,180]

There are dynamic changes in the cytokine milieu of the brain after stroke, suggesting that different types of T-effector cells and regulatory T cells could emerge depending on the timing of the lymphocyte-antigen encounter. Experimental data indicate that endogenous regulatory T cells are upregulated after stroke.[181,182] There are limited data on the development of T-effector cells after stroke but, under healthy conditions, T-helper-1 cells directed towards the brain (ie, myelin basic protein) are distinctly unusual.[181] If an animal has a systemic inflammatory stimulus at the time of stroke, however, the situation changes. For example, lipopolysaccharide, a component of the gram-negative bacterial cell wall and a Toll-like receptor 4 agonist, predisposes animals to develop a T-helper-1 response to myelin basic protein.[181] This effect of lipopolysaccharide is probably multifactorial, but upregulation of costimulatory signals on antigen-presenting cells and an increase in interferon γ secretion could be important. This autoimmune response is associated with worse stroke outcome, which suggests at least one explanation for why individuals who develop an infection after stroke have worse outcome.[164]

Clinical use of preconditioning

Preconditioning has been successful as an experimental procedure to identify mechanisms for brain protection and regeneration. Important examples of strategies to modulate these mechanisms include erythropoietin, activators of mitochondrial K_{ATP} channels, and volatile anaesthetics.

Because of the high risk of neurological complications associated with coronary artery bypass grafting and carotid endarterectomy, patients scheduled for these procedures could potentially benefit from therapeutic preconditioning. These complications include stroke and cognitive deficits, which result either from haemodynamic compromise or embolism (both

macroembolism or microembolism).[183,184] However, late cognitive decline after coronary artery bypass grafting is not specific to the use of cardiopulmonary bypass. Characteristics of patients, particularly the degree of pre-existing vascular disease in the brain, might have a more important role for patients undergoing these procedures.[185] Nevertheless, neuroprotective strategies such as preconditioning, or the use of mimetics or preconditioning effectors could be initiated before surgery. The approach might be useful in subarachnoid haemorrhage because about 20-30% of patients have delayed neurological ischaemic deficits associated with vasospasm several days after the initial event.[186] Similarly, up to 10% of patients with transient ischaemic attack will have a stroke within a month of the event.[187]

By contrast with conventional neuroprotection trials, in which neuroprotective drugs are given after the ischaemic event, preconditioning strategies have the advantage of antagonising the deleterious mechanisms at the earliest possible time, because the intervention precedes the injury. Moreover, a baseline assessment of neurological status before patients have an ischaemic insult is included (figure 1). The disadvantage of such a clinical trial strategy, which has been proposed as a promising approach to provide clinical proof of concept for neuroprotection studies in stroke research,[188] is that a large sample size is needed because of the overall low rates of neurological complication.

Several clinical trials are underway to test the safety and efficacy of preconditioning strategies for protecting the brain against anticipated damage. For example, one randomised controlled trial aims to determine whether remote ischaemic preconditioning (by thigh cuff inflation; see below) decreases subclinical cerebral and myocardial damage in patients undergoing carotid endarterectomy.[189] A pharmacological preconditioning strategy (ie, preloading) is being tested by treating patients with subarachnoid haemorrhage with erythropoietin α to prevent the delayed ischaemic neurological deficits and their consequences.[190] Several trials have been or are assessing post-treatment of cerebral ischaemia with preconditioning effectors such as erythropoietin,[191] nitric oxide,[192,193] and interleukin 1 receptor antagonist.[194]

Preconditioning is an invaluable technique in basic neuroprotection research and lends itself to new designs of clinical trials. Short episodes of spontaneous ischaemia (such as angina or transient ischaemic attack) might induce endogenous protection against subsequent longer and potentially deleterious episodes of organ ischaemia. For example, when an acute myocardial infarction is preceded by preinfarction angina, the resultant infarction is milder, with fewer cardiac arrhythmias and better left ventricular function than those without preceding angina.[195] This observation has motivated the therapeutic use of brief balloon inflations before longer coronary interventions to protect the heart (early preconditioning approach) and promoted the development of delayed preconditioning mimetic compounds such as nitro glycerin.[196] Both strategies have been successfully tested in randomised controlled trials.[196,197] Data from two retrospective studies of stroke showed that in patients with previous transient ischaemic attack the severity of subsequent stroke was attenuated and the outcome better than those without a preceding event.[198,199] In a small prospective study, despite similar size and severity of the perfusion deficit, patients with previous transient ischaemic attack had smaller initial diffusion lesions and final infarct volumes and showed clinical deficits that were less severe than in those without a preceding event.[200] Together with other clinical evidence,[201,202] these observations suggest that endogenous preconditioning triggered by a transient ischaemic attack is present in the human brain. A criticism of these studies, however, is that patients with previous transient ischaemic attack might fundamentally differ from those without, particularly with respect to cause of stroke. This problem is further exacerbated by the heterogeneity of stroke and the people who have it; furthermore, some transient ischaemic attacks are associated with MRI evidence of infarction and others are not.[203] Two other retrospective studies showed no association between previous transient ischaemic attack and low stroke severity.[204,205]

Therefore, whether the clinical event of a transient ischaemic attack is neuroprotective and associated with less brain injury from subsequent strokes is unclear.

Clinical use: challenges and opportunities

A central belief in preconditioning research is that the preconditioning stimulus must be sub-threshold and should not cause damage. The postulated dose response of the preconditioning stimulus therefore ranges from no response at low intensities to the protected state at higher intensities; a further increase in stimulus intensity will cause overt damage. The therapeutic range of preconditioning is narrow.[4,71] Most preconditioning studies are short in duration, with limited periods of survival (up to 1 week); these studies might therefore have missed the maturation of damage. Furthermore, few studies have used techniques sensitive enough to detect subtle structural injury. Increasingly, sophisticated brain imaging suggests that transient ischaemic attack (ie, a potential preconditioning equivalent) commonly leads to structural damage to the brain.[203] Sommer[206] postulated that "ischemic PC [preconditioning] necessarily involves some form of brain damage, leading to functional impairment with behavioral deficits, although without any lesion". If true, titration of the preconditioning stimuli so that it has an effect but does not cause damage to the tissue might not only be difficult but potentially impossible. Whether preconditioning mimetics, such as mild inhibition of mitochondrial respiration by aspirin[35] or activation of mitochondrial K_{ATP} channels,[207] are non-toxic is unclear. Because of these potential problems, preconditioning effectors could be a safer way to provide endogenous brain protection than preconditioning mimetics or preconditioning itself. Another caveat is that medication such as anti-inflammatory drugs or statins could interfere with signalling cascades involved in the induction of preconditioning and therefore could diminish (or even potentiate) protective responses to preconditioning.

Immunological preconditioning and other interventions that modulate the immune response to induce neuroprotection and enhance repair pose specific challenges. First, the immune response can stimulate recovery at more delayed time points after stroke;[208] therefore, prolonged immunomodulation could interfere with these important restorative processes. Most animal studies focus on early stroke outcomes (days) and might therefore miss important intervention effects weeks to months after treatment. Second, most immunomodulatory drugs are non-specific and affect the immune response in the brain and elsewhere. Because patients with stroke are already predisposed to infection,[209] drugs that might further increase this risk are of limited benefit. The strategy of inducing immunological tolerance to CNS antigens is appealing[210] because this approach would lead to an immunomodulatory response only where the antigen is present and accessible to the immune system, thus localising the effects of the therapy to the brain during times of compromise of the blood-brain barrier. However, studies of mucosal tolerance for the treatment of multiple sclerosis show that slight differences in antigen treatment could affect both the efficacy and safety of this strategy. For example, mucosal delivery of antigen can give rise to a T-helper-1 response to that antigen. Such a response could potentially lead to encephalitis, as was seen in the vaccine trials of amyloid β for Alzheimer's disease.[211] However, if the CNS immune response was completely suppressed, the susceptibility to infection such as progressive multifocal leukoencephalopathy might increase, as was seen in the studies of natalizumab for multiple sclerosis.[212,213]

Search strategy and selection criteria

References for this Review were identified through searches of PubMed by use of search terms that included "preconditioning", "ischemic tolerance", "neuroprotection", "brain repair", "brain ischemia", "brain hypoxia", and "stroke" ("tolerance" and "preconditioning" were common modifiers), with various search periods (from January, 1980, to December, 2008). The full list of search terms is available from the author on request. The

bibliographies of the most recent articles were also screened to fi nd other previously unidentified articles. Only papers published in English were reviewed. Further references were obtained from personal reprint files.

Additional factors need to be understood before considering preconditioning for clinical application, even if safe strategies are identified. For example, preconditioning stimuli and effectors in the neuroprotective signalling cascades might be specific to sex,[44,214] dependent on age,[215-217] and affected by diet[110,218] and medical comorbidities.[219,220] Because the target population for preventive neuroprotection is usually elderly patients with several medical problems, investigators who undertake preclinical studies are advised to take these factors into consideration.

Despite these challenges, several promising strategies to elicit endogenous brain protection are under clinical development. As patients' safety is the main concern, compounds or strategies that are already in use for other indications in which they have shown safety are highly attractive candidates. For example, HIF1 mediates the activation of a large cassette of genes involved in the adaptation to hypoxia and ischaemia.[47] The iron chelator desferrioxamine is clinically approved for various indications, including thalassaemia and other iron-overload syndromes. Erythropoietin is approved for treatment of anaemia and seems safe and effective for critically ill patients who are anaemic and have had trauma.[221] Various inhalational anaesthetics used in human beings (eg, sevoflurane) induce preconditioning and tolerance against brain ischaemia and act as brain protectants after ischaemia in preclinical experiments.[43,222,223] These compounds are safe and effective in eliciting early preconditioning in patients undergoing coronary artery bypass graft surgery in randomised controlled trials.[224] These drugs also elicit delayed preconditioning in human beings.[225,226] Another promising approach in clinical testing is the induction of early remote preconditioning in patients undergoing carotid endarterectomy. In a randomised controlled trial, patients who receive ischaemic preconditioning have a thigh cuff inflated on one leg until flow in the pedal arteries stops.[189] After 5 min the cuffis moved to the opposite thigh. The cycle is repeated so that each leg has two 5-min periods of ischaemia followed by 5 min of reperfusion. Another attractive concept, albeit still in an early clinical development stage, is the induction of mucosal tolerance (immunological preconditioning) against E-selectin via a nasal spray to lower stroke events and reduce the effect of cerebral ischaemia.[227]

Conclusions

Preconditioning (ie, induced tolerance) is an experimental technique in which protective and regenerative mechanisms of the brain can be isolated from deleterious mechanisms. The molecular signalling cascades of endogenous brain protection—from stimulus and sensor to transducers and effectors—are being identified (figure 2). Research has led to the discovery of several promising strategies for the treatment of patients with acute CNS injury. Additionally, studies of preconditioning have led to the preloading concept of preventively inducing protection in patients who are at high risk of damage to the brain in the near future. Preconditioning-derived strategies include haemopoietic cytokines, immunological tolerance, and physical measures such as remote ischaemic preconditioning. Some of these interventions seem to be safe and effective in protecting the ischaemic heart, and they are now being tested in randomised clinical trials to protect the brain. However, there are still many uncertainties about this therapeutic approach, including which doses of preconditioning or mimetics are safe and effective, and whether transient ischaemic attacks are an ischaemic preconditioning equivalent in human beings. Even in cardiology, where preconditioning strategies have been clinically studied and applied for more than a decade, conclusive evidence for the efficacy and safety of preconditioning or the use of mimetics is scarce. Consequently, therapeutic guidelines

for the treatment of heart disease do not yet advocate its use, and there is still little clinical evidence for the use of preconditioning to protect the brain. Such basic factors need to be resolved before randomised controlled trials of preconditioning for the treatment of cerebral ischaemia can be done.

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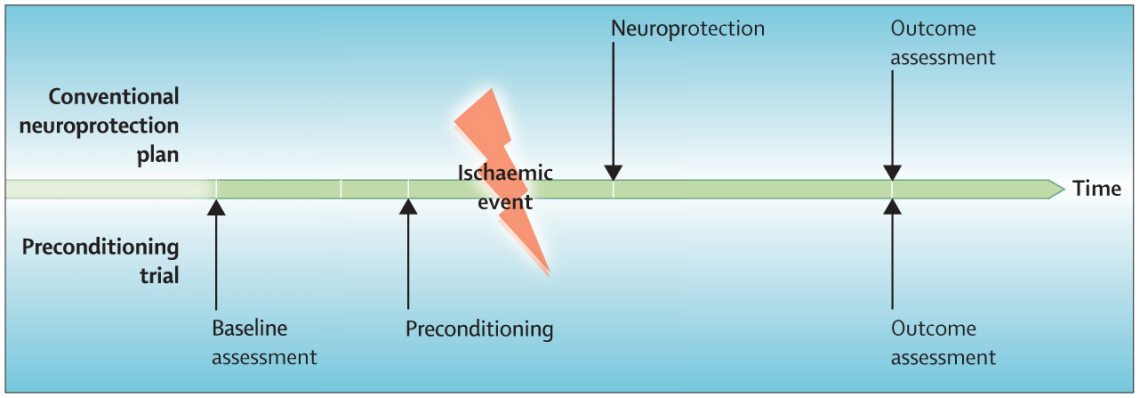

Figure 1. Comparison of conventional and preconditioning neuroprotection trials

Note that the preconditioning trial enables a complete baseline assessment and leads to organ protection before ischaemia (or trauma).

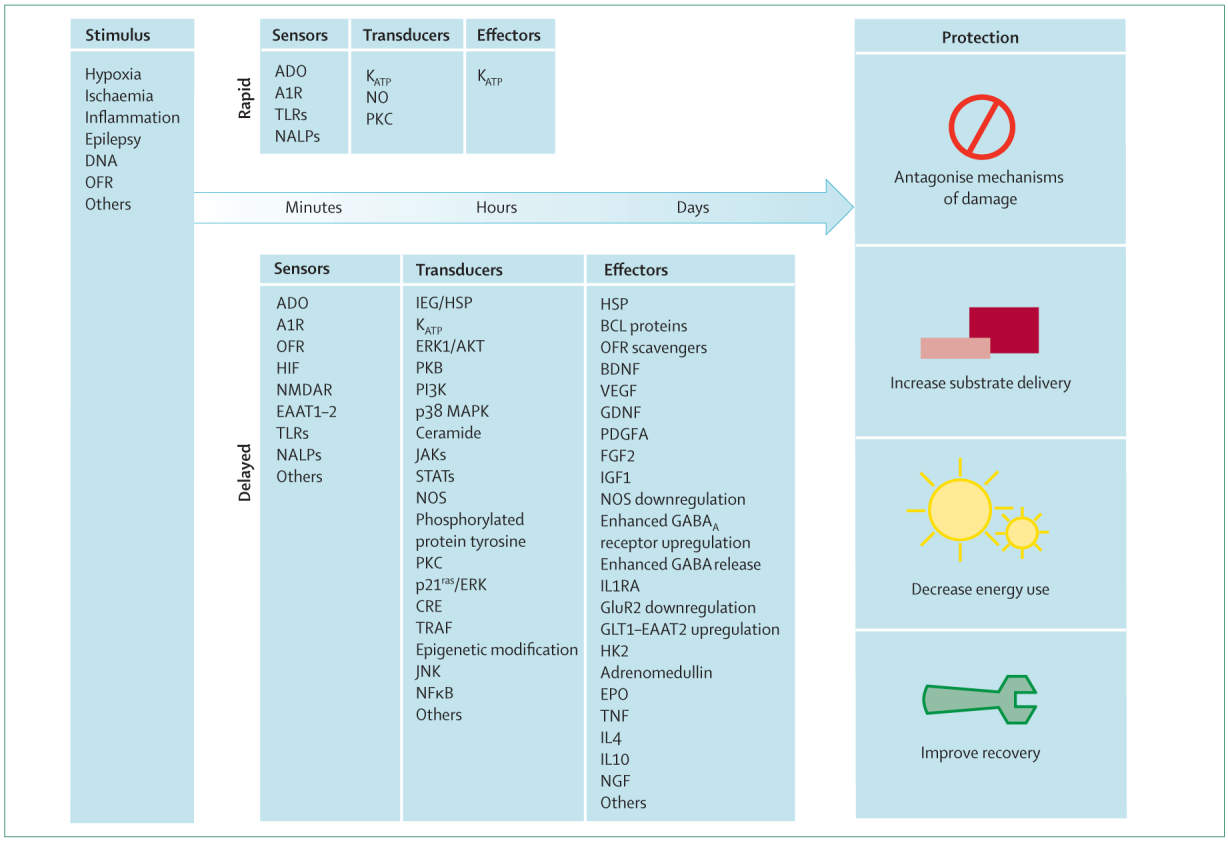

Figure 2. Signalling cascades of preconditioning

Various stimuli lead to protection via modules of sensors, transducers, and effectors. Adapted from Dirnagl et al,[4] with permission from Elsevier. A1R=adenosine receptor type 1. ADO=adenosine. Akt=a serine/threonine kinase family. BDNF=brain-derived neurotrophic factor. CRE=cyclic AMP response element. EAAT=excitatory amino-acid transporter. EPO=erythropoeitin. ERK=extracellular signal-regulated kinase. FGF2=fibroblast growth factor 2. GDNF=glia-derived growth factor. GluR2=glutamate receptor subunit 2. GLT=glutamate transporter. HIF=hypoxia-inducible factor. HK2=hexokinase 2. HSP=heat shock protein. IEG=immediate early gene. IGF1=insulin-like growth factor 1. IL=interleukin. IL1RA=interleukin 1 receptor antagonist. JAK=janus kinase. JNK=c-Jun N-terminal kinase. K_{ATP}=ATP-sensitive potassium channel. MAPK=mitogen-activated protein kinase. NALP=NACHT-containing, LRR-containing, and pyrin-domain-containing protein. NFκB=nuclear factor κB. NGF=nerve growth factor. NMDAR=NMDA receptor. NO=nitric oxide. NOS= nitric oxide synthase. OFR=oxygen free radicals. PDGFA=platelet-derived growth factor receptor A. PI3K=phosphoinositide-3 kinase. PKB=protein kinase B. PKC=protein kinase C. STAT=signal transducer and activator of transcription.TNF=tumour necrosis factor. TLR=Toll-like receptor. TRAF=TNF receptor-associated factor. VEGF=vascular endothelial growth factor.

Table

Types of preconditioning

	Principle	Example
Cross	Preconditioning stimulus is different from the noxious stimulus against which it protects	In the earliest description of the technique[20] the same stimulus was used to induce preconditioning and damage (trauma), whereas trauma was used by Janoff [21]to induce protection against sterile sepsis and bowel ischaemia
Remote	Preconditioning of one organ or system leads to protection of a different (remote) organ	The prototypical approach for remote preconditioning is the initiation of short ischaemic intervals to a limb to protect organs such as the heart[22,23] and brain.[24-26] Remote preconditioning might indicate a crosstalk between the brain and the rest of the body in response to stress. Randomised clinical trials have already shown the efficacy of this strategy for the heart.[27,28] Remote preconditioning is a particularly attractive strategy to protect organs that are highly susceptible to damage but that are difficult to target, such as the brain
Immunological	Pharmacological compounds that trigger the signalling cascades of preconditioning (without a physical stimulus)	One of the earliest examples of pharmacological preconditioning was the mild inhibition of the respiratory chain with nitropropionic acid or acetylsalicylic acid,[35-38] which induced cellular changes such as those seen with hypoxia. Another relevant example is the use of the iron chelator desferrioxamine,[39] which induces nuclear translocation of the transcription factor hypoxia-inducible factor 1, with consequent expression of a plethora of hypoxia-inducible genes, including erythropoietin, vascular endothelial growth factor, and hexokinase
Anaesthetic	Short application of any one of many different classes of anaesthetics can induce an ischaemia-protected state	Anaesthetic preconditioning is effective in the heart[40,41] and brain.[42-45] This protection might happen within minutes or might be delayed by many hours or days. For practical reasons, anaesthetic preconditioning is an appealing therapeutic option that has already been tested in randomised controlled trials for cardiac protection
Mimetics	Compounds that emulate the main danger signal can lead to preconditioning	Examples include inhibitors of mitochondrial respiration, stabilisers of hypoxia-inducible factor 1 (signal: hypoxia), or lipopolysaccharide (signal: infection)[46,47]
Effectors	The downstream mediators of protection	These effectors include proteins such as erythropoietin, vascular endothelial growth factor, and BCL family proteins. These proteins are attractive pharmacological candidates for preventing the consequences of anticipated ischaemia