Preconditioning and the Developing Brain

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Preconditioning occurs when a subinjurious exposure renders the brain less vulnerable to a subsequent damaging exposure. In this essay, various models of preconditioning in the immature brain are discussed. Adenosine, excitatory amino acids, nitric oxide, hypoxia-inducible factor, ATP-sensitive K\textsuperscript{+} channels, caspases, heat shock proteins, inflammatory mediators and gene expression all seem to be involved in sensing, transducing and executing preconditioning resistance. Also reviewed in this essay is evidence that some subinjurious exposures render the brain more vulnerable to a subsequent damaging exposure. We believe that unraveling the mechanisms of how the developing brain becomes inherently resilient or vulnerable will offer important insights into the pathogenesis of injury. Preconditioning of the brain or induction of tolerance of the immune system might be utilized in the future to decrease CNS vulnerability and the occurrence of perinatal brain injury.

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In light of recent advances in the field of preconditioning (PC), we consider it appropriate to review this area of research. Elucidating the endogenous mechanisms involved in PC might provide information that can guide us to a better understanding of the critical events leading to brain injury. This, in turn, would help us design prophylactic and therapeutic interventions. Because our major goal is to find ways to protect the developing brain, we have emphasized PC effects in the immature central nervous system.

Background and Definition

In a seminal paper published in 1986, Murray and his colleagues reported that the extent of myocardial infarction resulting from a sustained coronary occlusion was diminished if the heart had been subjected previously to brief periods of sublethal ischemia.\textsuperscript{1} This modulation of a response to an otherwise lethal exposure by a preceding sublethal exposure defines PC. Similar PC effects on the heart have been observed in humans.\textsuperscript{2,3} One report suggests that transient ischemic attacks (TIA) induce ischemic PC in the brain.\textsuperscript{4}

PC, also sometimes known as tolerance, occurs in a number of organs, can be induced by different sublethal insults, and can occur at remote sites. For example, ischemia in one cerebral hemisphere can induce tolerance in both hemispheres to subsequent forebrain ischemia.\textsuperscript{5} Moreover, this concept of “remote PC” can be extended to include PC of one organ protecting another organ.\textsuperscript{6} This suggests that a circulating substance is involved in communicating the PC message to other organs. Although early studies used the same PC exposure as the potentially toxic exposure, subsequent studies documented that PC to a toxic exposure could be achieved with exposure to subtoxic levels of a different exposure, so called cross-tolerance.\textsuperscript{7,8} Usually PC means that a prior sublethal exposure renders the tissue less sensitive to the severe insult. In this sense, PC protects the organ. Some recent studies, however, have demonstrated that a sublethal exposure (eg, to lipopolysaccharide or “in vitro ischemia”) increases vulnerability to further insults.\textsuperscript{9} In this sense, PC, or perhaps more appropriately, “negative PC,” is a sensitizing factor (Fig. 1).

Here we limit the expression PC to illustrate the reduction in the brain’s vulnerability made possible by a previous subthreshold exposure (chemicals, toxins, hypoxia, epilepsy, cytokines or any other exposure). We use the term sensitization to describe a situation where the brain is rendered more vulnerable by a previous subthreshold exposure.
The interaction between a sub-threshold insult and a severe insult may result in decreased brain injury, so called preconditioning. In contrast, the term sensitization describes a situation where the brain is rendered more vulnerable by a previous sub-threshold exposure. (Color version of figure is available online.)

Cellular Biology of Preconditioning in the Brain

Models of PC in Immature Animals

A number of different insults can induce PC in adult animals, including hypoxia, ischemia, inflammatory cytokines, endotoxin, spreading depression, seizures, excitotoxins, hyperthermia and mitochondrial toxins. Usually, a certain time period (typically 12-72 hours) between PC and the severe insult is required to achieve tolerance. For example, induction of hyperthermia (41.5-42°C for 15 minutes) 6 to 24 hours before hypoxia–ischemia conferred a considerable reduction of brain injury in postnatal day 7 rats. Longer lasting protection is suggested by the observation that transient (30 minute) intrauterine ischemia 12 days before an hypoxic–ischemic insult on postnatal day 7 still increased neuronal survival from 44% to 74%. On the other hand, the salutary effects of PC are partly lost when the interval between the threshold exposure and evaluation is prolonged (eg, weeks), especially in young animals. Although no study has demonstrated that the benefits of PC are permanent, they do seem to last for weeks, if not months. In immature rats, 3 hours of exposure to 8% oxygen, 24 hours before a prolonged hypoxic–ischemic insult, reduces brain injury by 70 to 90%, which persists up to 3 weeks. Indeed, we recently found that the structural damage and the functional deficits following the severe insult were still markedly (by 72%) reduced after 8 weeks of recovery in the PC compared with the sham control group.

Mechanisms of PC

Acquisition of tolerance appears to depend on stress sensors, signal transduction and effectors of protection (Fig. 2). Stress sensors (or proximal triggers) detect various stressful conditions and convert the information into an intracellular stress response. The signal is thereafter processed through the signal transduction system, activating various effector systems that will reduce the vulnerability of the cell or tissue. Single factors can sometimes act both as a sensor and part of the cellular signal transduction/effector system (eg, HIF-1, caspases and nitric oxide).

Adenosine

PC stimuli (eg, hypoxia or ischemia) disturb the energy balance resulting in an accumulation of adenosine. The subsequent activation of adenosine A1 receptors seems to be a critical early step in tolerance. A1 receptor antagonists inhibit PC protection in a number of tissues, including the adult CNS. The role of adenosine A1 receptors has not been explored in PC models of immature animals and may actually be less critical as A1 receptors do not appear to function during the neonatal period.

Glutamate

Glutamate is also released in response to PC insults, leading to activation of both N-methyl-D-aspartate (NMDA) and alpha-aminooxy-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Indeed, PC can be induced by administration of glutamate receptor agonists. NMDA receptor agonists block tolerance in the adult brain, but not in the immature brain. Preconditioning might also prevent the downregulation of GluR2 AMPA type of receptors, which would decrease intracellular calcium overload. In the immature white matter, the situation appears to be quite the opposite, as sublethal exposure to oxygen–glucose deprivation down-regulates GluR2 receptors, increases Ca2+ uptake and increases the vulnerability of oligodendroglial precursor cells. This is an example of the sensitization (“negative PC”) we refer to above.

Nitric Oxide (NO)

NO may be involved in hypoxic PC in the immature brain. In one study of 7-day-old rats, the nonspecific NOS inhibitor L-nitroarginine almost completely inhibited PC protection. Specific inhibitors of the neuronal and inducible isoforms of NOS, however, had no effect on the PC response, indicating the importance of endothelial NOS (eNOS).

Hypoxia Inducible Factor 1 (HIF-1)

HIF-1 is a heterodimeric transcription factor composed of HIF-1β, which is constitutively expressed, and HIF-1α, which is tightly regulated by the oxygen concentration. Consequently, HIF-1α, is rapidly induced by hypoxia in the neonatal brain and may thereby serve as a stress sensor. HIF-1 may actually also function as an effector to promote cell survival by inducing genes that contain a hypoxia response element that includes binding sites for HIF-1. In the immature brain, also induce HIF-1 expression. Indeed, hypoxic PC induces the expression of a number of HIF-1 target proteins (or mRNAs) like glucose transporter 1, aldolase, phosphofructokinase, lactate dehydrogenase, erythropoietin and vascular endothelial growth factor that may make the brain resistant to further insults.

Both desferrioxamine and CoCl2, each known to protect the immature brain (see below), also induce HIF-1α expression. However, HIF-1 target genes expressed after hypoxia were not induced by CoCl2 and there is currently no direct evidence yet that HIF-1 is responsible for PC protection in the immature brain.
ATP-Sensitive K⁺ Channels, Caspases and Heat Shock Proteins

ATP-sensitive K⁺ channels, which may be important at the signal transduction/effector level, can be activated by adenosine A1 receptors, oxygen free radicals and through a complicated kinase cascade involving both the protein kinase C family and mitogen-activated protein kinases.20 Channel blockers inhibit PC and channel openers can induce tolerance in many systems.19,20 The mechanisms are partly unresolved, but activation of these channels in the plasma membrane leads to hyperpolarization that may prevent or delay detrimental depolarizations. Alternatively, opening of ATP-sensitive K⁺ channels in mitochondria may dissipate the potential across the inner membrane that would prevent wasteful ATP-hydrolysis or increase mitochondrial buffering of cytosolic Ca²⁺.20 Another possibility is that opening of ATP-sensitive K⁺ channels leads to a limited release of mitochondrial cytochrome C and activation of caspase-3, which will turn on compensatory systems like the heat shock proteins (HSPs). HSPs are intracellular molecular chaperones of naïve, aberrantly folded or mutated proteins, but they also prevent the assembly of the apoptosome and inactivate caspase-3.32,33

Such a sequence of events may be relevant for the immature brain because administration of an ATP sensitive K⁺ channel opener (diazoxide) protects against hypoxia–ischemia.34 Heat shock protein 72 is induced in response to stimuli that promote PC10 and caspases play a key role in immature cell injury.35-37 Preconditioning-induced activation of caspase-3 may also cleave and inactivate poly(ADP-ribose)polymerase-1 (PARP-1), which would add to the tolerant state as activation of PARP-1 enhances brain injury also in the immature brain.38,39

Preconditioning and Gene Expression

In most cases a certain time lag (typically 12-72 hours) is required between the PC stimulus and the severe insult to obtain PC protection (at least for the brain). This has led to the inference that protein synthesis is needed for PC. This inference is supported by the observation that the protein synthesis inhibitor cycloheximide inhibits ischemic PC30 and by the evidence that proteins play an important role in PC (including such transcription factors as HIF-1, nuclear factor kappa B (NF-kB), ceramide, activator protein-1, early growth response gene-1 and cAMP response element-binding protein).31,42

Preconditioning insults

hypoxia, ischemia, proinflammatory cytokines, endotoxins, spreading depression, seizures, excitotoxins, hyperthermia, mitochondrial toxins

Stress sensors

ATP-sensitive K-channels; NMDA receptors (?), i.e. Ca²⁺, NO, adenosine A1 receptors (?), oxygen free radicals; activation of caspase-3; Heat shock proteins;

Signal transduction

<table>
<thead>
<tr>
<th>Kinases</th>
<th>Transcription factors</th>
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<tbody>
<tr>
<td>PKC</td>
<td>NFkB</td>
</tr>
<tr>
<td>Raf-1, P42/p44(Erks), p38, p21ras, MEKs, JNK/SAP</td>
<td>Jak-2</td>
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<tr>
<td></td>
<td>ceramide</td>
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<td></td>
<td>HIF-1a</td>
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<tr>
<td></td>
<td>CREB</td>
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Effectors of protection

| Altered synthesis of proteins or posttranslational modification | heat shock protein 72 & 27 | eNOS      |
| Hypoxia-inducible factor | Erythropoietin | MnSOD     |
| ATP sensitive K channels (mitochondria) | Bcl-2-family of proteins | metallothionein-1 and-2 |
| HIF-1a target genes     | 12-lipoxygenase     | MKP-1     |
| Preservation of mitochondrial function | heme oxygenase-1 | block of GluR2 (?) |

Figure 2 Acquisition of tolerance depends on stress sensors, signal transduction and effectors of protection. Stress sensors detect various stressful conditions and convert the information into an intracellular stress response. The signal is thereafter processed through the signal transduction system, activating various effector systems that will reduce the vulnerability of the cell or tissue.
A number of these genes/proteins, besides HIF-1 target genes and heat shock proteins, are expressed after PC. Based on their specific properties, some of them may be anticipated to act as intermediates between the PC stimulus and the observed protection. For example, metallothionein-1 and -2 are expressed in the immature brain after hypoxic PC and even though the functions of these proteins are unknown, they are protective in models of ischemia. Furthermore, MAP-kinase phosphate-1 (MKP-1) mRNA, which is induced after hypoxia, improves survival by antagonizing c-Jun N-terminal kinase (JNK) activation, a contributor to CNS injury. A considerable number of other genes are also induced by the PC process (e.g., lipoxigenases, cytokines, chemokines, etc.). With the increased availability of micro-arrays, we expect that more will be identified.

**Vascular Effects: Cerebral Blood Flow**

Although some PC-related phenomena lead to the inference that improved cerebral blood flow accounts for some of the PC effects, studies in the adult CNS have shown that PC does not affect cerebral blood flow during or after ischemia. In addition, the fact that PC can be induced in vitro provides additional support for the view that factors unrelated to blood flow are at play. No studies have been published on PC and cerebral blood flow in the immature brain. However, in an immature (6-day-old) rat model of 2.5 hours of systemic hypoxia followed 24 hours later by the same systemic hypoxia accompanied by cerebral ischemia, it was observed protection. For example, metallothionein-1 and -2, which are up-regulated following sublethal ischemia in vivo, are induced following hypoxia alone. These results suggest an effect on cerebral blood flow or a direct effect on energy metabolism.

**Cytokines**

Under certain circumstances pro-inflammatory cytokines afford protection against cerebral ischemia. Both IL-1β and TNF-α are up-regulated following sublethal ischemia in vivo. Ischemic preconditioning appears to be dependent on TNF-α release as inhibition of TNF-α convertase (TACE/ADAM17, which is involved in TNF-α release), blocked both the increase in TNF-α and the neuroprotective effects of preconditioning. Pretreatment of hippocampal cell cultures with TNF-α induces protection against subsequent oxidative insults such as FeSO4 or amyloid b-peptide exposure. TNF-α pretreatment also protects against focal cerebral ischemia in mice, however, only when given intracisternally. Furthermore, both TNF-α and IL-1β protect neurons in culture against subsequent hypoxic or excitotoxic injury. In the adult gerbil, repeated administration of IL-1 over several days protects hippocampal neurons from subsequent ischemic injury, an effect that is blocked by coadministering of IL-1 receptor antagonist (IL-1ra).

In neuron cultures, the protective effects of TNF-α appear to reflect activation of NF-kB with subsequent induction of manganese superoxide dismutase (Mn-SOD) and suppression of both peroxynitrite formation and apoptosis. Nuclear translocation of NF-kB plays a central role following several PC stimuli as inhibition of NF-kB activation abolishes the preconditioning effects of sublethal ischemia, as well as epileptic and polyunsaturated fatty acid insults. In astrocyte cultures, TNF-α-induced tolerance is associated with the inhibition of the phosphorylation of the NF-kB subunit, p65/RelA, resulting in suppression of the ICAM-1 gene, while other NF-kB dependent genes such as Mn-SOD are not affected. These results therefore suggest that cytokine-induced tolerance is associated with different mechanisms in different cell types.

Other possible downstream beneficial effect of the induction of TNF-α and IL-1β is the production of neurotrophins, which are markedly induced by pro-inflammatory cytokines and by PC. In support of neurotrophins as PC intermediaries, is the observation that intracerebral administration of BDNF for 7, 10 or 14 days before ischemia reduces infarction volume, without affecting CBF. BDNF may exert its protective properties via crosstalk between the TrkB receptor and the NMDA receptor. Thus, under certain circumstances and at relatively low levels, pro-inflammatory cytokines appear to participate in a physiologic stress response that contributes to the development of tolerance in adult animals and in vitro. The role of cytokines in PC in the immature brain is complex and still unclear. Cytokines play important roles in the developing brain, are easily induced following ischemia, and might contribute to damage. We await evidence that cytokines and neurotrophins are involved in PC in the immature brain.

**Lipopolysaccharide (LPS)**

In the adult, the endotoxin LPS and the endotoxin analog diphosphoryl lipid A confer tolerance to cerebral ischemia. LPS priming that increases tolerance against focal cerebral ischemia could be due to its stimulation of cytokines, such as TNF-α expression. Furthermore, ceramide, a down stream messenger of TNF-α signaling, contributes to LPS-induced tolerance to cerebral ischemia. PC induced by LPS is also associated with the induction of TGF-β1, which has neuroprotective properties.

LPS pretreatment does not appear to affect the cerebral blood flow immediately after MCA occlusion, but may diminish the severity of secondary microvascular perfusion deficits. In support of this concept, endothelial NOS is up-regulated following LPS and administration of the NOS inhibitor L-NAME abolishes the preconditioning effects of LPS. Other studies suggest that up-regulation of antioxidants (superoxide dismutase) following low doses of endotoxin may be important in LPS-induced tolerance.

To our knowledge, endotoxin-induced PC has not been studied in the immature brain. We have recently found that LPS sensitizes the immature rat brain. Repeated daily doses of LPS, in utero, have been shown to induce cardiovascular tolerance but without cerebral protection in the fetal sheep. These observations suggest that effects of LPS priming may be variable depending on the physiological state, the presence of certain risk factors for vascular injury and age.
Brain Injury Protection Analogies to PC

Chemical Protection

Some chemicals that protect the immature brain might do so by means other than those that characterize PC. Thus, we are not yet prepared to consider glucocorticoids, desferrioxamine and cobalt chloride as “chemical” preconditioners. Nevertheless, some of the similarities deserve attention. For example, glucocorticoids do not provide brain protection against hypoxia/ischemia if given immediately prior, during or after the insult in immature or adult animals. However, if dexamethasone, is given 4 to 24 hours before hypoxia–ischemia, brain injury is prevented in 1 to 2 weeks old, but not in 4-week-old pups demonstrating that not only the interval between the protector and the insult can be critical but also the age of the animals. The relatively long half-life of glucocorticoids would allow their effects to be exerted in the brain for days after administration and their presence might therefore contribute to a direct protective effect. Thus, a PC like mechanism needs not to be invoked, although it remains a possibility.

As previously mentioned, HSPs are seen as protectors of the cell after exposure to stressful stimuli and may have a role in PC. The primary mediator of the heat shock response is the heat shock transcription factor 1 (HSF1). When HSF1 binds to one of the HSPs, a conformational switch occurs that rapidly activates HSF1 in a manner that mimics the kinetics of glucocorticoid receptor pathways mediated by cochaperones. Thus, steroid receptor function might be analogous to some phenomena presumed to be similar to some PC processes.

Other examples of “chemical” preconditioners in immature animals are desferrioxamine and CoCl2. Giving either of these substances 24 hours before hypoxia–ischemia in 7-day-old rats reduces brain injury by more than 50%. Desferrioxamine has a half-life of only 3 hours in the rat. Desferrioxamine is present in myelin basic protein. For Alzheimer disease, the antigen has been amyloid-beta (Aβ) protein. In animals studies intended to reduce infarct size, the antigens have been myelin basic protein, ovalbumin, and selectin.

These tolerized cells become a form of regulatory T cell called Tr3 cells, which secrete cytokines such as IL-10 and transform growth factor-β1 on antigen restimulation. These Tr3 cells thereby modulate the brain-damaging inflammatory response by “active cellular regulation” or “bystander suppression.”

PC-induced tolerance differs considerably from immunization-induced tolerance. Nevertheless, they can be viewed as analogous, at least in that both are prophylactic procedures intended to diminish the effects of insults that would otherwise lead to brain cell death. We offer this detail about immunization-induced tolerance because it is a possible alternative to PC or a supplemental therapeutic approach. This alternative/supplement might be especially attractive if circulating inflammatory cells are shown to adversely affect the immature brain.

Immunization

Immunization is defined as the induction of an immune response that is beneficial to the host in halting a pathological process. The earliest, and most current, immunizations promote the organisms’ ability to respond vigorously to an infectious organism (eg, H influenza). When an inflammatory response is damaging, however, the goal should be to turn down the ability to respond vigorously to a stimulus. Progress has been made recently in efforts to induce tolerance to CNS components, thereby promoting the development of regulatory/suppressor cells that modulate subsequent potentially damaging inflammatory phenomena.

Antigen(s) can be injected, although risk of adverse may be heightened. An alternative approach exposes the animal or person to the antigen(s) via the nose or mouth. Nasal- and gut-associated lymphoid tissues are thought to have evolved to prevent the host from reacting to inhaled or ingested proteins that are nonpathogenic. T cells in the nose and bowel can be made tolerant with a low-dose regimen of a CNS antigen. For multiple sclerosis, the preferred antigens have been synthetic polypeptides whose components are present in myelin basic protein. For Alzheimer disease, the antigen has been amyloid-beta (Aβ) protein. In animals studies intended to reduce infarct size, the antigens have been myelin basic protein, ovalbumin, and selectin.

Concluding Remarks

Although PC does occur in the immature brain, very few investigators have addressed this topic. The complexity of PC needs to be much better understood before PC is considered a viable prophylactic approach to prevent brain damage in the newborn. For example, the possibility exists that a PC stimulus intended to protect the brain might instead sensitize the brain to an insult. On the other hand, the potential for PC to reduce the occurrence/severity of brain damage in the most vulnerable humans is sufficient reason to explore this topic in greater detail. Perhaps chemical preconditioners can be found that do not have this sensitizing capability. Other possibilities to protect the brain may be to induce tolerance in circulating inflammatory cells as activation of these are known to adversely affect the immature brain. Might such immuno-modulating interventions help to minimize perinatal brain damage in the future?

Acknowledgments

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