

BRAIN TOLERANCE AND PRECONDITIONING

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In this review article the authors describe a phenomenon of “brain tolerance” which represents transient resistance of brain tissue to a lethal insult established by preconditioning with a mild insult of short duration. Tolerance evoked by brief ischemia resembles transient ischemic attack(s) (TIA) often preceding full-blown ischemic stroke in a clinical setting. A series of recent studies have described another relevant phenomenon termed “chemical preconditioning”. Several substances interfering with cellular energy metabolism applied in subtoxic doses may provide protection against lethal insults of a different type. For example, 3-nitropropionic acid (3-NP), antibiotics erythromycin and kanamycin, acetylsalicylic acid, and 2-deoxyglucose have been shown to evoke tolerance. Recently, we have reported that NMDA receptor antagonists and 2-deoxyglucose used at relatively low doses were potent agents to potentiate the protective anticonvulsant effect induced by transient brain mild ischemia. Further studies are expected to prove similar action of these drugs in other experimental models. Based on the accumulated experimental and clinical data the brain tolerance subsequently reinforced by pharmacological intervention might become a successful prophylactic strategy against serious brain insults in patients.

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The term "brain tolerance" describes a phenomenon of transient resistance to a lethal insult evoked by preconditioning with a mild insult of short duration. Preconditioning stimulus, while inadequate to damage neurons, somehow increases their ability, and sometimes the ability of the whole organism as well, to survive subsequent lethal challenge. The first description of brain tolerance was provided by Kitagawa et al. [28, 29]. These authors found that preconditioning with a brief period of global ischemia protected hippocampal neurons from necrotic damage by severe global ischemia, provided that the preconditioning stimulus lasted for not less than 2 min and the lethal insult was applied not earlier than after 24 h. As further studies have shown, also transient focal ischemia establishes tolerance against subsequent global ischemic damage, both within the region of the preconditioning ischemic zone [15] and in ipsilateral hippocampal neurons [38]. Furthermore, preconditioning by a mild global ischemia significantly decreases infarct size after permanent middle cerebral artery occlusion [56] and a similar phenomenon has been observed following a brief reversible focal ischemia [1]. The aforementioned four experimental paradigms of ischemic tolerance induction are referred to as the global-global, focal-global, global-focal and focal-focal model, respectively [7]. The ischemic tolerance usually is evident 24 h after the preconditioning stimulus, and it lasts for 5–7 days.

Brain tolerance can also be established by, as well as it can protect against various non-ischemic lethal events. For example, hypoxic preconditioning protects neonatal rats against subsequent ischemia [14]. Spreading depression produces ischemic tolerance in rat brains [30, 36, 37]. A short ischemic insult attenuates behavioral effects of, and decreases mortality caused by the pharmacological activation of NMDA receptors in mice [22]. Transient whole body hyperthermia protects rat brain against subsequent ischemic damage [9]. Ischemic tolerance has also been described following preconditioning with hypothermia [43] and with a low dose of lipopolysaccharide [3].

Another relevant phenomenon is termed "epileptic tolerance". Sasahira et al. [53] reported that brief seizure episodes protected against neuronal injury by severe seizures induced later. It has also been found that hypoxic preconditioning attenuates brain edema associated with kainic acid-induced status epilepticus in rats [13]. Transient bilateral

occlusion of carotid arteries (BCCA) in rats reduced susceptibility to seizures evoked by hippocampal kindling [4], bicuculline [55] or pilocarpine [21]. The epileptic tolerance seems to be a longer lasting phenomenon than the ischemic tolerance.

In our studies on adult mice, preconditioning with 30-min BCCA reduced mortality after pilocarpine- or bicuculline-induced toxic challenge 14 days after the preconditioning stimulus [45–48].

A series of recent studies have described another related phenomenon termed "chemical preconditioning". Several substances interfering with cellular energy metabolism applied at subtoxic doses may provide protection against some lethal insults. For example, Riepe et al. [51] reported that preconditioning with a specific inhibitor of succinic dehydrogenase, 3-nitropropionic acid (3-NP) evokes hypoxic tolerance in rat hippocampal slices. Similar effects of 3-NP were observed *in vivo* when brain ischemia was used as a lethal challenge [32, 41]. Preconditioning potential is also a property of antibiotics erythromycin and kanamycin [23], acetylsalicylic acid [50] and 2-deoxyglucose (2-DG, a non-metabolizable analogue of glucose) [63].

The development of tolerance following various sublethal insults is a feature shared by many tissues and organs including heart and skeletal muscle, lung, kidney, liver, intestine, retina, spinal cord and brain [52 and the references cited therein]. Although marked organ-specific differences, such as the difference between brain and heart, exist [58], some mechanisms of tolerance may be universal. A prominent tissue-unspecific mechanism, which may be employed in the creation and maintenance of tolerance, is the induction of stress proteins known as the heat shock proteins (HSPs), of which the most extensively studied is the HSP70 family. HSPs are proteins which function as "molecular chaperones" assisting polypeptide chains in establishing functional conformation, and which are also involved in degradation and reactivation of damaged proteins, in intracellular trafficking, nuclear receptor binding and some other "cellular house-keeping" jobs [44, 61]. Some HSPs are expressed constitutively, other are induced in a stereotypical cellular response to external stress, which is highly conserved through yeast, plants, insects and mammals [61].

There is a strong, albeit correlative evidence for the neuroprotective role of HSPs. It has been repeatedly shown that following brain ischemia the

survival of brain neurons is positively correlated with the expression of HSP70. Thus, it seems likely that cells overexpressing HSPs are more resistant to lethal damage. How do these stress-induced proteins protect cells? It is probable that HSP70 and other HSPs (such as HSP27, HSP90 and heme oxygenase-1) express pleiotropic cytoprotective effects [61]. These may include improving stability of cellular antioxidative enzymes such as superoxide dismutase resulting in the attenuation of cellular oxidative damage during and after the lethal challenge [61 and the references cited therein]. Also, in non-neuronal cells, overexpression of HSP70 mRNA attenuated the influx of Ca^{2+} by desensitizing other ion-exchanging systems [27 and the references cited therein], and prevented heat- or ceramide-induced apoptosis through caspase inhibition [40].

Although induction of stress proteins in response to cellular stress is a general biological phenomenon, some aspects of cytoprotective action of HSPs may be brain-specific. It is generally accepted that ischemia as well as severe seizures induce a specific chain of events in the brain, leading to neuronal death known as the "excitotoxic cascade". This cascade is triggered by the abundant release of stimulatory neurotransmitter glutamate to the extracellular space and excessive activation of neuronal N-methyl-D-aspartate (NMDA) and non-NMDA ionotropic glutamatergic receptors [8, 59, see also 17]. A dominant source of glutamate flood appears to be the reversal of excitatory amino acid transporters (EAATs). These transporter proteins clear glutamate from extracellular space under normal conditions, but when transmembrane ionic gradients are disrupted (which is the case during ischemia, as well as during severe seizures), the increased intracellular Na^+ bind to and induce the reversal of glutamate flow [57]. Excitotoxic effects of glutamate on neurons are mediated by glutamate receptors, and executed by various intracellular effector mechanisms. These include the increase in intracellular calcium level leading to the activation of kinases, phospholipases and proteases, followed, *inter alia*, by destruction of membrane phospholipids, proteolytic cleavage of structural proteins and protein denaturation [8]. Neuroprotection attributed to HSPs may result from their ability of binding proteases, phospholipases, kinases and neurotransmitter receptors with resultant attenuation of neurodestructive effects [31].

Overexpression of HSPs may be a decisive event in the establishment of brain tolerance. Global ischemia lasting 3 to 8 min evoked HSP70 synthesis in neurons and in some glial cells after 24 h, and increased expression of this protein lasted for 7 days [35], closely resembling the aforementioned temporal scheme of brain ischemic tolerance in the global-global model. After preconditioning with focal ischemia neuronal expression of HSP70 preceded the onset of tolerance to the lethal focal ischemia, but its duration was compatible with that of tolerance [6]. The stimulation of HSP70 synthesis in brain cells has also been observed following kainic acid-induced seizures [64] and after subtoxic doses of 2-DG [63]. Interestingly, the development of ischemic tolerance has been inhibited by intracerebroventricular application of either a non-selective inhibitor of HSP70 expression quercetin, or anti-HSP70 antibodies [42].

Besides the induction of HSP, changed expression of other proteins may also be involved in brain tolerance. After global cerebral ischemia, the apoptosis effector gene *bax* was up-regulated in the CA1 layer of the hippocampus where the most vulnerable neurons reside, while the anti-apoptotic gene *bcl-2* was expressed in CA3 layer which contains neurons much less susceptible to ischemic insult [6]. A shift of *bcl/bax* balance toward an anti-apoptotic phenotype observed following chemical preconditioning with 3-NP has been suggested as a potential explanation for brain tolerance in this paradigm [5]. In a focal-focal ischemia model of ischemic brain tolerance, the increase in interleukin-1 receptor antagonist (IL-1ra) protein has been found [1], and this result corresponds to known neurotoxic properties of IL-1b [60] and neuroprotective properties of IL-1ra [49]. Another mechanism which may contribute to the development of brain tolerance has recently been proposed on the basis of the finding that preconditioning with cortical spreading depression down-regulates EAATs with the time frame similar to that reported for tolerance [10]. Last but not least, in the focal-focal ischemia model tolerance to brain injury was associated with the reduced poststroke expression of early response genes *c-fos* and *zif268* [1], suggesting that tolerance-related modifications of protein expression occur not only before, but also during the final challenge.

The mechanisms underlying the development of brain tolerance are not yet fully elucidated. Short

periods of ischemia may in some circumstances decrease brain tolerance toward subsequent lethal ischemia [25], but there is no simple explanation for this fact since a similar phenomenon has not been demonstrated in other organs. This may be the brain-specific mechanism of glutamate excitotoxicity, which in some circumstances compromises the development of tolerance by tissue-unspecific mechanisms, but such a hypothesis requires experimental proof. Also, while there is a substantial amount of data supporting the concept that HSPs expressed after preconditioning are neuroprotective, this issue is not conclusively solved. It has been repeatedly shown that inhibition of *de novo* protein synthesis by cycloheximide reduces neuronal loss in both global [16, 54] and focal [11, 34] ischemia. This effect has been attributed to the requirement of protein synthesis for the execution of post-ischemic apoptotic neuronal death, and interpreted as the evidence that proteins expressed following transient ischemia are neurotoxic rather than neuroprotective. A possible explanation could be that mild insults induce neuroprotective proteins (HSPs and possibly other), while proteins required for programmed cell death are induced only by more severe insults. However, certain early response genes (eg. *c-fos*) are induced by milder brain tissue depolarization than that required to induce HSPs. The threshold for HSPs reaction, in fact, seems to be closely approaching the threshold for neuronal injury [30, 37]. How to reconcile this phenomenon with the observation that tolerant brains display decreased expression of early response genes in response to ischemia?

When the brain-specific mechanisms of tolerance are considered, epileptic tolerance is a special case. As already mentioned, it may be evoked by temporary incomplete ischemia (such as BCCA) and it is longer lasting than ischemic tolerance. After the BCCA preconditioning we have found increased GABA content in tolerant brains two weeks after the preconditioning insult. This is suggestive of the fact that the stimulation of inhibitory GABAergic neurotransmitter system may be an important contributor to the development and maintenance of epileptic tolerance [45]. Another important issue is the involvement of NMDA receptors in the development of the epileptic tolerance. This problem can be studied by pharmacological blockade of these receptors. However, although NMDA antagonists are typically considered as neuropro-

tectants, they do have neurotoxic potential as well. Non-competitive NMDA receptor antagonists such as dizocilpine caused pathomorphological damage in the retrosplenial cortex of the rat brain, and they also increased the expression of HSP-70 [19]. Moreover, drugs of this group induced neuronal apoptosis in cortical cultures [24]. Similar effects were attributed to competitive antagonists of NMDA receptors [12, 18, 20].

We have examined the effect of a competitive NMDA receptor antagonist CGP-40116 on the epileptic susceptibility after BCCA. Systemic administration of this drug given at two doses of 4.0 mg/kg *ip*, one given 1.5 h before and the other 6 h after BCCA, diminished but not abolished the anticonvulsant effect of the preconditioning insult observed 14 days later [48]. We have also found that a non-competitive NMDA antagonist MK-801 (dizocilpine) administered at a dose of 1 mg/kg in similar experimental paradigm failed to inhibit the protective effect of BCCA against bicuculline toxicity [45, 46]. This stands in contrast with some previous studies in which NMDA antagonists completely abolished tolerance to severe ischemia in the brains preconditioned by a transient and mild insult [2, 26]. However, our results may indicate that the anticonvulsant effect after BCCA is mediated by particular subtypes of NMDA receptors [33], or by some other excitatory amino acid receptors which co-participate in the development of tolerance. In particular, the role of AMPA receptors should be considered [62]. Interestingly, the prolonged treatment with low doses of MK-801 or CGP-40116 markedly potentiated the anticonvulsant effect of transient brain ischemia [45, 46, 48]. These data show for the first time that tolerance evoked by ischemic preconditioning may be potentiated by pharmacological treatment.

In our most recent study, we have assessed the influence of repetitive subtoxic doses of 2-DG on the protective effect induced by 30-min BCCA against susceptibility to bicuculline-evoked seizures and bicuculline-related toxicity in mice. The lethal challenge was applied 14 days after the preconditioning stimulus. Additionally, we have examined the effect of protein synthesis inhibitor cycloheximide (CHX) on the development of tolerance in these experimental paradigms. Our results showed that 2-DG alone did not influence epileptic susceptibility, but it reduced seizure-related mortality. BCCA combined with 2-DG resulted in a mar-

ked decrease in seizure susceptibility as well, as mortality. CHX abolished the antiepileptic effects of BCCA alone, as well as BCCA combined with 2-DG, whereas it did not influence the 2-DG-related decrease in mortality. We concluded that the development of BCCA-induced epileptic tolerance, as well as unmasking antiepileptic effects of 2-DG by BCCA, was dependent on protein synthesis, but seizure-related mortality was reduced by 2-DG through another mechanism, which was independent of protein synthesis [47].

A large interest in brain preconditioning and tolerance stems from the relevance of these phenomena to some clinical situations. Tolerance evoked by brief ischemia resembles transient ischemic attack(s) (TIA) preceding full-blown ischemic stroke in a clinical setting. Some authors say that in animal models preconditioning provides better protection of brain against ischemia than any pharmacological treatment currently available. Indeed, according to the result of a recent analysis, TIAs are neuroprotective. Patients who encountered ipsilateral TIA before cerebral infarction, on average recovered better compared to those who did not have TIA [39]. If brain tolerance can be reinforced by some pharmacological treatment, a reasonable strategy would be to start a pharmacological intervention either after transient ischemic attack (TIA) to potentiate the endogenous protection against the next more severe insult, or as a prophylaxis in patients severely threatened by stroke (eg. those scheduled for carotid endarterectomy). After all, when patients are given antibiotics or acetylsalicylic acid, are their brains preconditioned against ischemia or seizures?

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