Molecular Physiology of Preconditioning-Induced Brain Tolerance to Ischemia

TIHOMIR PAUL OBRENOVITCH

Division of Pharmacology, School of Life Sciences, University of Bradford, Bradford, United Kingdom

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Ischemic tolerance describes the adaptive biological response of cells and organs that is initiated by preconditioning (i.e., exposure to stressor of mild severity) and the associated period during which their resistance to ischemia is markedly increased. This topic is attracting much attention because preconditioning-induced ischemic tolerance is an effective experimental probe to understand how the brain protects itself. This review is focused on the molecular and related functional changes that are associated with, and may contribute to, brain ischemic tolerance. When the tolerant brain is subjected to ischemia, the resulting insult severity (i.e., residual blood flow, disruption of cellular transmembrane gradients) appears to be the same as in the naive brain, but the ensuing lesion is substantially reduced. This suggests that the adaptive changes in the tolerant brain may be primarily directed against postischemic and delayed processes that contribute to ischemic damage, but adaptive changes that are beneficial during the subsequent test insult cannot be ruled out. It has become clear that multiple effectors contribute to ischemic tolerance, including: 1) activation of fundamental cellular defense mechanisms such as antioxidant systems, heat shock proteins, and cell death/survival determinants; 2) responses at tissue level, especially reduced inflammatory responsiveness; and 3) a shift of the neuronal excitatory/inhibitory balance toward inhibition. Accordingly, an improved knowledge of preconditioning/ischemic tolerance should help us to identify neuroprotective strategies that are similar in nature to combination therapy, hence potentially capable of suppressing the multiple, parallel pathophysiological events that cause ischemic brain damage.

I. INTRODUCTION

Preconditioning/ischemic tolerance of the brain, heart, and other organs refers to a natural adaptive process that can be induced by a variety of sublethal insults (e.g., transient hypoxia), and which increases the tissue tolerance to a subsequent, potentially lethal ischemia. This adaptive cytoprotection is a fundamental capability of living cells, allowing them to survive exposure to potentially recurrent stressors. Preconditioning induces two different time windows of tolerance. The first phase (often named “rapid preconditioning”) occurs rapidly af-
ter the stimulus used for induction of preconditioning and only lasts for ~1 h, whereas the delayed phase corresponds to a robust state of tolerance that is usually detectable 24 h after preconditioning induction, peaks at 3 days, and fades after 7 days. For clarity sake, in this review, “preconditioning” describes which initial stimulus or treatment (e.g., chemical preconditioning) was used for the induction of increased resistance to ischemia and/or other stressors. “Ischemic tolerance” is restricted to define the period of increased resistance of the brain to ischemic injury, whereas the terms brain tolerance or tolerant brain define the wider notion of increased cerebral resistance to ischemia and other stressors.

Clearly identified in the heart by Murry et al. in 1986 (262), preconditioning and subsequent ischemic tolerance were then demonstrated in the brain (203), and attracted rapidly the interest of clinical and basic neuroscientists for several reasons. First, this biological process became widely recognized as a pertinent and effective experimental probe to understand how the brain protects itself against ischemia, thereby providing an innovative approach for the discovery of novel cerebroprotective strategies. Second, retrospective case-control studies showed a clinical correlate of the phenomenon discovered experimentally. Patients with a history of transient ischemic attack (TIA) have a decreased morbidity after stroke (284, 432). Finally, other findings suggested that brain tolerance may be induced, or mimicked pharmacologically (39).

This review focuses on brain tolerance which, as defined above, lasts several days after preconditioning induction. Its purpose is to provide an integrated account of the adaptive modifications that are associated with brain tolerance, with emphasis placed on effector mechanisms (i.e., the molecular and related functional changes that may increase tolerance). In all studies, preconditioning is used to induce a state of tolerance prior to the subsequent, potentially lethal “test” ischemia (i.e., a procedure equivalent to a neuroprotective pretreatment) and, therefore, the molecular changes underlying ischemic tolerance may be effective against deleterious processes that occur during or after the lethal ischemia. This review was written for investigators who are new to this field of study, and to help experts including and interpreting their findings within a more complex and integrated picture. Several previous reviews provide a broader coverage of brain preconditioning and ischemic tolerance (94, 136, 201), and readers should refer to those for information on complementary issues such as 1) Which molecular sensors are activated by preconditioning and initiate the biological responses that ultimately lead to ischemic tolerance? 2) Which signaling cascades and transducers contribute to the development of ischemic tolerance?

As outlined in the table of contents, alterations occurring at the cellular level and their functional consequences constitute section II. Changes in synaptic transmission, which may also alter brain tolerance, are assembled in section III. Finally, changes in mechanisms that involve multiple systems (e.g., inflammation) constitute section IV. This organization is imperfect at times, because some biological changes (e.g., mitochondrial preservation) are involved in several mechanisms that may underlie ischemic tolerance. Indeed, it has become clear that ischemic tolerance results from interrelated, multifactorial processes, and this key feature is the primary rationale for this review. The complexity of the biological responses underlying ischemic tolerance may reflect a fundamental reprogramming of the brain tissue genomic response to injury, a notion proposed by Stenzel-Poor, Simon, and colleagues from their microarray analyses of changes in gene expression (386, 387). In accordance with the purpose of this review, subsections covering topics that have been extensively investigated (e.g., inflammation and ischemic tolerance) are less comprehensive and detailed than previous reviews dedicated to the corresponding topic; such more specialized reviews are cited whenever appropriate.

In the numerous studies reviewed herein, preconditioning was induced by stressors as diverse as brief period(s) of hypoxia or ischemia, cortical spreading depression, and administration of bacterial lipopolysaccharides (LPS). Therefore, it is pertinent to outline here the notion of cross-tolerance, i.e., one stressor induces tolerance against a different stressor (127, 201, 316). The efficacy of cross-tolerance, relative to the classic ischemic preconditioning (i.e., brief, sublethal cerebral ischemia, with subsequent test ischemia that would damage the naïve brain; Ref. 372) may be more modest, and it appears to vary with the nature and intensity of the first challenge (201). The window of ischemic tolerance may be also shifted or different; for example, the “tolerizing” effect or LPS needed more time for maturation, starting at 48 h after LPS injection and reaching full potential at 72 h in the study of Tasaki et al. (407). For more details on cross-tolerance, ischemic tolerance dependency on de novo protein synthesis, and kinetics of increased brain resistance to ischemia induced by preconditioning, see Kirino (201).

There are two notes of caution. Cross-tolerance is an important feature, but potentially misleading by suggesting that different preconditioning stimuli result ultimately in similar mechanisms of tolerance. That may be only partially true, and dependent on the nature of the stimuli considered. Comparison of the genomic profiles of brain responses to ischemia from mice preconditioned with either brief ischemia or low-dose LPS revealed that a substantial subset of the differentially expressed genes were unique to each preconditioning stimulus (385). This suggests that the nature of the preconditioning stimulus may determine a specific neuroprotective phenotype (385).
When a specific variable (gene expression, level of a specific protein, etc.) is found to be altered in animals subjected to both preconditioning stimulus and test insult, this change may be causally related to the mechanism of protection, and this interpretation is often favored by authors. However, as the fundamental nature of preconditioning is to protect the brain against a subsequent insult, an alternative and valid interpretation may be that the identified change simply reflects, or is an epiphenomenon of, the reduced damage. This potential pitfall should be kept in mind.

II. CELLULAR “HOUSE- AND HEALTH-KEEPING” MECHANISMS IN THE TOLERANT BRAIN

A. Cellular Ionic Homeostasis and Energy Metabolism

As brain preconditioning implies the induction of biological processes prior to a potentially lethal insult, it is rational to examine whether brain cell tolerance to ischemia is actually strengthened during the test insult. Among other mechanisms, this could be achieved by improved brain energy metabolism under low oxygen and/or glucose, or a prolonged latency for anoxic depolarization after the ischemia onset. With regard to the latter, cellular depolarization is clearly detrimental to the ultimate outcome in models of cerebral ischemia. 1) Ischemic neuronal damage subsequent to transient global ischemia or hypoxia correlates closely with the duration of anoxic depolarization (20, 187, 283, 374), and 2) the size of infarct in stroke models increases with the number and total duration of peri-infarct depolarizing events (250, 408). In addition, brain cells appear to have the ability to adapt to hypoxia by reducing their energy demand through modulation of ion channels, which delays anoxic depolarization (150, 162, 419, 437). Reduced brain energy demand is also a feature of hibernation and hypothermia, two other conditions that reduce the brain vulnerability to energy deprivation (22, 118).

In mice subjected to preconditioning alone (induced by 15 min of middle cerebral artery occlusion, MCAO) or followed 3 days later by damaging ischemia (60-min MCAO), the profile of changes in gene expression suggested metabolic depression and attenuation of ion-channel activity (387). Follow-up experiments with cultured rat cortical neurons showed that preconditioning induced by brief noninjurious oxygen and glucose deprivation decreased voltage-gated potassium currents (387). However, most of the data reviewed next do not support the notion that reduced energy demand and ion channel “arrest” are determinant factors for ischemic tolerance.

1. Ischemic tolerance and latency of anoxic/ischemic depolarization

When brain preconditioning was produced by a standardized ischemic insult to the forebrain (i.e., transient 4-vessel occlusion to produce anoxic depolarization for 2–3.5 min) in rats, at the time for maximum neuroprotection in these experiments (i.e., 2 days postpreconditioning), anoxic depolarization was delayed by ~1 min during the subsequent ischemic insult (417). This indicated that the severity of the subsequent test insult was reduced slightly by preconditioning in this rat model, but such an effect was not observed when the same experimental strategy was applied to gerbils (1), nor in mice with cortical spreading depression (CSD) preconditioning and bilateral carotid occlusion as subsequent test insult (Godukhin OV, Jackson G, Obrenovitch TP, unpublished data). Furthermore, other data in the rat study suggested that the delayed anoxic depolarization in the preconditioned group only played a minor contribution toward ischemic tolerance, because the ischemic thresholds for hippocampal neuron loss, measured 1 wk after the test insult, increased from 4 min of ischemic depolarization in controls to 15 min in the preconditioned group, i.e., far more than the 1-min increase of the anoxic depolarization latency (417). Finally, the anoxic depolarization latency was not significantly different between slices from gerbils subjected to ischemic preconditioning and those from sham-operated gerbils, when these slices were exposed to anoxia (191). The experimental strategy used in this latter study was pertinent because it ruled out a potential, interfering cerebrovascular effect of preconditioning (see sect. nB).

2. Ischemic tolerance and peri-infarct depolarizing events

Electrical stimulation of the cerebellar fastigial nucleus (FN) for 1 h was found to elicit a marked and long-lasting increase in the brain tolerance to insults, with features suggesting that the resulting tolerance was similar to that achieved by other preconditioning methods: 1) effective protection against different insults, including global and focal ischemia (141, 333) and excitotoxicity (139); 2) delayed protection against MCAO was maximal 3 days after FN stimulation; 3) decreased susceptibility of brain cells to apoptosis in vitro (462); and 4) decreased inflammatory reactivity in the brain (129). However, the most interesting finding within the context of this subsection was that FN stimulation at 3 days before MCAO also markedly reduced the development of peri-infarct depolarizations (142). It would be pertinent to test whether this effect occurs with other preconditioning methods, because peri-infarct depolarizations are known to contribute to lesion progression in MCAO models (250, 408) as well as in humans with acute brain injuries (107).
addition, as these events occur early after ischemia onset, their suppression could contribute significantly to the reduction of the lesion volume that can be detected within hours of permanent MCAO (e.g., when measured at 6 h after MCAO in mice previously subjected to CSD-preconditioning; Godukhin et al., unpublished data). As proposed by Golanov et al. (142), this effect of FN stimulation may be linked to a reduction of neuronal excitability, a hypothesis in line with some previous data indicating that preconditioning (hypoxic, ischemic, or hyperthermic) decreases the susceptibility to seizures evoked by bicuculline, kainic acid, or pentylentetrazol (101, 320, 345). However, in two other studies, increased resistance to kainic acid excitotoxic neuronal damage was not associated with reduced susceptibility to kainic acid-induced seizures (193, 316).

3. Cellular ionic homeostasis in the ischemia-tolerant brain

Ischemic preconditioning, induced 24 h before global forebrain ischemia in rats, prevented the inhibitory effect of ischemia/reperfusion on Na+/K+-ATPase activity in cytoplasmic membrane fractions of hippocampus and cerebral cortex (92), but this was probably a consequence of cytoprotection as N-methyl-D-aspartate (NMDA) receptor block with dizocilpine (MK-801) had similar effects (258). Given the potentially lethal consequences of intracellular Ca2+ overload (32), it is relevant to examine whether Ca2+ homeostasis is altered in ischemic-tolerant brain cells. When ischemic preconditioning preceded a lethal 5 min of forebrain ischemia in gerbils, several potentially beneficial changes were found in hippocampal CA1 neurons, including the following: plasma membrane Ca2+-ATPase activities were elevated before the test ischemia, and remained subsequently at a higher level, and mitochondrial sequestration Ca2+ was enhanced (285). In line with these data, imaging of intracellular Ca2+ ([Ca2+]i) showed that its elevation in hippocampal CA1 neurons after an anoxic-aglycemic episode was markedly inhibited in the ischemia-tolerant gerbil (367). Furthermore, also in the hippocampus of gerbils, the expression of the plasma membrane Ca2+-ATPase isoform 1 (PMCA1) was increased, together with that of inducible 72-kDa heat shock protein (HSP70), when brain ischemic tolerance was enhanced by 3-nitropropionic acid preconditioning (190). It is relevant to mention that the Na+/Ca2+ exchanger (NCX) gene was upregulated after transient global ischemia in rats (235). However, the duration of the recirculation period was only 6 h in this study (235), and more recent experiments suggested that NCX gene expression after permanent MCAO in rats is regulated in a differential manner, depending on the exchanger isoform (NCX1, -2, or -3) and the region involved in the insult (i.e., ischemic core, peri-infarct areas or spared regions) (42, 313).

Na+/H+ exchange and Na+/K+-2Cl− cotransport are also critical ion transporters as they contribute to the regulation of intracellular pH and cell volume (305). This dual role, however, makes it difficult to predict whether a change in the efficacy of these transporters may promote or impair the survival of cells to ischemia; for example, enhanced Na+/H+ exchange would help to reduce intracellular pH but promote cell swelling and further Ca2+ influx through Na+/Ca2+ exchange. Comparison of cortical astrocyte cultures from wild-type and Na+/H+ exchanger isoform 1 deficient [NHE1(−/−)] mice suggested that NHE1 deficiency actually attenuated the disruption of ionic regulation and swelling induced by 2 h of oxygen and glucose deprivation (200), and this notion was confirmed in vivo (234). In wild-type mice treated with the Na+/H+ exchange inhibitor HOE 642 and in NHE1(−/−) mice, the size of infarct measured 24 h after 2h of MCAO was reduced by around one-third. (65). Similar data were obtained with genetic manipulations of the Na+/K+-2Cl− cotransporter isoform 1 (NKCC1) in mice and inhibition of NKCC1 by bumetanide (65). According to these data, one could expect ischemic tolerance to be associated with a downregulation of NHE1 and/or NKCC1, but no report of such an association could be found for either central nervous system (CNS) or myocardium.

4. Glucose consumption, ATP levels, and lactic acid accumulation/clearance

As the delay for anoxic/ischemic depolarization is, to some extent, related to the cerebral metabolic rate during the ischemic insult (270, 425), one would not expect a major change in the latter variable to be associated with ischemic tolerance. This notion was supported by the elegant studies of Maruoka and co-workers (239, 291), in which the cerebral glucose metabolic rate (CMRglu) was measured serially in different regions of freshly prepared rat brain slices by using [18F]2-fluoro-2-deoxy-D-glucose as tracer. Compared with controls, 3 days after either hypoxic or 3-nitropropionic acid preconditioning, CMRglu in the frontal cortex was similar before and during the 20-min test hypoxia. After reoxygenation, there was a much better recovery of CMRglu in slices prepared from preconditioned animals, but this probably reflected increased cellular viability. Similarly, ATP and phosphocreatine levels in the cortex and hippocampus of gerbils were not changed 24 h after ischemic preconditioning (2-min bilateral carotid occlusion) or during the subsequent 5-min test ischemia and at 1-day reperfusion (189). However, conflicting data were obtained with immature rats: brain glycogen was increased 24 h after hypoxic preconditioning, and residual ATP measured at the end of the
90-min test hypoxia-ischemia was significantly higher than in controls (48).

Given the rapid increase in brain tissue and extracellular lactate levels, not only during ischemia (121, 232) but also with peri-infarct depolarizations (164), an improved ability to use lactate as an energy substrate would be a potentially important adaptive change of the brain energy metabolism, especially during reperfusion. Some studies suggest that the CNS has this capability and that it may be induced by preconditioning. 1) Neuron-rich slices (i.e., incubated in the presence of the glial toxin, fluorocitrate) prepared from the rat brain left hemisphere, of which the contralateral MCA was occluded 48 h before slice preparation, were able to utilize lactate as energy substrate, in contrast to slices of control rats (205). 2) The expression of monocarboxylate transporter 1 (MCT1, involved in the uptake and release of lactate, pyruvate and ketone bodies; 312) was increased in cultured primary rat astrocytes after 1-day hypoxia (423) and under inflammatory conditions (209). However, ischemic preconditioning in adult gerbils had no effect on lactate levels at 24 h postpreconditioning, during the test ischemia and up to 1 day within the reperfusion period (189).

Overall, these data do not suggest that an adaptation of brain energy metabolism, or increased latency for anoxic depolarization, can significantly account for ischemic tolerance. However, the possibility that the tolerant brain may have an improved ability to control intracellular Ca\(^{2+}\) overload deserves further investigations.

5. Preservation of mitochondrial membrane potential and function

Several studies in vitro and in vivo showed that brain ischemic tolerance is associated with preservation of mitochondrial function. Preconditioning by transient, sublethal exposure to cyanide protected primary cultures of chick embryonic neurons from neurotoxicity induced 24 h later by a more severe exposure to cyanide, and this effect was associated with better preservation of the mitochondrial membrane potential (178). These data were confirmed with cultured hypothalamic neurons when hypoxic preconditioning preceded anoxia (436). When nonsynaptosomal mitochondria prepared from the hippocampus of rats were assessed 24 h after 10 min of global ischemia, the rate of oxygen consumption and the activities of complexes I–IV were preserved by ischemic preconditioning induced 48 h before the test ischemia (88). These in vitro data were complemented by two similar studies in rats. Zhan et al. (453) reported a better preservation of mitochondrial cytochrome c during reperfusion in preconditioned animals; Zhang et al. (455) reported confirmatory data with MCAO as test insult. Supporting data were also obtained in gerbils (273). As the enhanced stability of mitochondrial membrane potential was associated with an overexpression of the anti-apoptotic Bcl-2 and Bcl-xL genes in the studies with cultured neurons (178, 436), it was proposed that the preservation of mitochondrial function may be a consequence of Bcl-2 overexpression (see sect. II.E). However, opening of mitochondrial ATP-dependent K\(^{+}\) channels (mK\(_{ATP}\) channels) emerges as a pertinent, alternative mechanism.

Localized in the inner membranes of mitochondria, mK\(_{ATP}\) channels are inhibited by ATP, ADP, long-chain CoA esters, and 5-hydroxydecanoate (5-HD), and their ATP inhibition is reversed by GTP, GDP, cromakalim, diazoxide, and other K\(_{ATP}\) channel openers. The primary role of mK\(_{ATP}\) is thought to be mitochondrial matrix volume regulation, with mK\(_{ATP}\) opening increasing the matrix volume (or preventing its contraction caused by mild mitochondrial depolarization) (18), but the physiological consequences of mK\(_{ATP}\) opening also include respiratory stimulation and matrix alkalization (79). The role of mK\(_{ATP}\) channels in cardioprotection is well documented, with robust evidence that the activation of these channels acts both as inducer and effector of heart ischemic tolerance (290). Although this issue has not been as intensively investigated in the CNS, brain mitochondria contain six to seven times more mK\(_{ATP}\) than heart mitochondria, brain and heart mK\(_{ATP}\) are regulated by the same ligands (18), and some data suggest that mK\(_{ATP}\) is also involved in brain ischemic tolerance. 1) 5-HD, a selective blocker of mK\(_{ATP}\), administered prior to preconditioning with 3-nitropropionic acid in rats, blocked the development of ischemic tolerance to focal cerebral ischemia (165), confirming and clarifying previous results obtained with the less-selective K\(_{ATP}\) channel blocker glibenclamide (158). 2) Pretreatment with mK\(_{ATP}\) channel openers (diazoxide or BMS-191095) reduced neuronal death produced by transient focal cerebral ischemia or venous ischemia in rats (245, 267, 368, 435). In the mouse MCAO model, diazoxide decreased markedly the cortical lesion and reduced neuronal apoptosis in the peri-infarct region, and this cerebroprotective effect was abolished by 5-HD (228). Diazoxide also protected hippocampal and cerebellar neurons from apoptosis induced by staurosporine, hypoxia, or oxidative stress (168, 228, 409). Note that although K\(_{ATP}\) channels are also present at the surface of cells (including neurons) and some experiments suggested that plasma membrane K\(_{ATP}\) channels also contribute to cytoprotection, the central role of mK\(_{ATP}\) is now widely accepted (290), and delayed ischemic preconditioning of hippocampal neurons was not altered in knock-out mice lacking the cellular SUR1-type K\(_{ATP}\) channels (260). More information on cell surface K\(_{ATP}\) channels is available in section II.D.

Although the cytoprotective mechanisms associated with increased mitochondrial K\(^{+}\) uptake subsequent to mK\(_{ATP}\) channel opening in the heart have already received much attention, the beneficial consequences of increased
mitochondrial K⁺ uptake during ischemia/reperfusion remain speculative (for review, see Refs. 12, 108, 131). These may include energy conservation subsequent to reduced ATP transport across mitochondrial membranes, attenuation/prevention of mitochondrial Ca²⁺ overload, changes in the mitochondrial generation of reactive oxygen species (ROS), and prevention of mitochondrial permeability transition (MPT, a key mechanism of mitochondrial injury shown to occur at reperfusion). Clearly, it is now timely to investigate how important are these mechanisms to brain ischemic tolerance.

B. Transport Systems Across the Cellular Membrane

1. Glucose transport

Brain glucose uptake and utilization involve primarily the glucose transporter proteins GLUT1 and GLUT3 (422). A high density of GLUT1 in a highly glycosylated form is present in cerebral vessels endothelium where it facilitates the diffusion of glucose across the blood-brain barrier. In the brain parenchyma, a less glycosylated form of GLUT1 is present in glia, whereas GLUT3 is essentially neuronal (148, 422). No study dedicated to the effect of preconditioning on brain glucose uptake could be found when this review was prepared, but several lines of evidence suggested that an upregulation of GLUT1 and GLUT3 may be associated with brain ischemic tolerance.

1) Even though blood-brain glucose transport is not the rate-limiting step of brain glucose metabolism under normal conditions, there is evidence that it can adapt to improve glucose delivery to neurons in situations such as reduced plasma glucose (hypoglycemia) or excessive synaptic activity (prolonged/repeated seizures) (223). In rats, chronic (i.e., 5- or 12- to 14-day duration) insulin-induced hypoglycemia produced significant increases in both GLUT1 mRNA and protein levels together with a redistribution favoring luminal transporters (212, 373). Studies with cultured capillary endothelial cells also showed that glucose deprivation enhanced GLUT1 gene expression, with maximum effect observed 24 h after glucose removal (40). There is also convincing in vivo and in vitro evidence that neuronal GLUT3 is upregulated when glucose availability is reduced (99, 219, 264, 418); for example, 3-day starvation in mice increased brain GLUT3 mRNA by twofold, and 2-day glucose deprivation increased that of primary neuronal cultures by fourfold (264). In line with these data, work with primary cultures of rat neurons showed that 24-h hypoxia rapidly increased neuronal GLUT1 and GLUT3 mRNA up to 40- and 5-fold, respectively, with similar changes in GLUT1 mRNA measured in glia (49). Interestingly, the same study showed that although glucose deprivation alone produced minimal effects on GLUT mRNA levels, hypoxia plus glucose deprivation synergized to markedly increase GLUT gene expression (49). With regard to excessive brain activity, in adult rats, chronic seizures produced by pentylentetrazole or kainic acid increased the expression of both GLUT1 with maximum abundance at 3 days (146). In contrast, GLUT-1 densities were significantly decreased in some structures of the visual cortex when rats were subjected to chronic visual deprivation (98). All the aforementioned studies relate to chronic imbalance between brain glucose supply and demand, but there is also evidence that glucose transport can be very rapidly upregulated. In mice, GLUT1 upregulation was evident in both hypothalamus and cortex 3 h after mild hypoglycemia induced by a single insulin injection (241). In adult rats treated with pentylentetrazole, acute upregulation of blood-brain barrier (BBB) glucose transport occurred within 3 min of an initial seizure (78).

2) Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates the adaptive response to hypoxia in mammalian cells (361, 362). As such, brain HIF-1 was found consistently upregulated early after hypoxic preconditioning (33, 34, 184, 185, 347), after reversible cerebral ischemia (181, 311), and in the peri-infarct penumbra (31). More relevant within the context of this subsection, GLUT1 is one of the HIF-1 target genes, together with erythropoietin (EPO) and vascular endothelial growth factor (VEGF) (362).

3) In line with the early induction of HIF-1 by preconditioning (point 2), differential gene expression analysis identified GLUT1 as an upregulated gene in samples of preconditioned brain relative to controls (34, 184, 406) and in the rat MCAO model penumbra (31). Interestingly, GLUT1 was also upregulated in hypoxic macrophages in vitro (50), but one can argue that this may be detrimental to brain cell survival during cerebral ischemia because macrophages accumulating in ischemic areas could “steal” an essential fuel away from neurons in vulnerable areas. Also relevant, especially within the context of LPS preconditioning, is the study of Cidad et al. (73), showing that incubation of cultured rat astrocytes with LPS for 24 h increased the level of constitutively expressed GLUT1 mRNA and triggered GLUT3 mRNA expression (that was absent in control astrocytes). In the same study, incubation of astrocytes for 4 h in the absence of glucose, or under an oxygen-poor atmosphere (3%), also upregulated GLUT3 mRNA.

Increasing the capacity of the transport of glucose to neurons appears to be an efficient way to enhance the resilience of the brain to ischemia, at least with regard to glucose transport across the BBB, because procedures that lead to GLUT1 overexpression, by themselves, were cerebroprotective. Virus-mediated GLUT1 overexpression enhanced glucose uptake in hippocampal cultures and in adult rat hippocampus (160); protected the cultured neurons against exposure to glucose deprivation, glutamate,
or the mitochondrial toxin 3-nitropropionic acid (161); and reduced kainic acid-induced neuronal damage in the rat hippocampus (218). Treatment of ovariectomized female rats with the estrogen 17β-estradiol, which is known to upregulate GLUT1 in the BBB (363), reduced the ischemic lesion produced by permanent MCAO (364) and extended the threshold for ischemic damage produced transient MCAO (109). 17β-Estradiol also protected cultured rat brain endothelial cells against anoxia and glucose deprivation (364). Note, however, that estrogen neuroprotection may involve several other mechanisms, besides GLUT1 upregulation (for review, see Refs. 45, 332). Against this experimental background, it is relevant to mention that women appear to be naturally more resistant to stroke during premenopausal life, and if hormone replacement therapy (HRT) may not be considered because of adverse effects, hormonal therapy in other forms may be suitable for neuroprotection in clinical practice (for review, see Refs. 45, 332). Some antiapoptotic systems also have the ability to upregulate GLUT1. When human neuroblastoma cells were exposed to low-glucose medium, treatment with insulin-like growth factor I (IGF-I) or Bcl-2 upregulated GLUT1, enhanced glucose transport, and improved neuronal survival (348).

To conclude, an improved capacity of the transport of glucose from blood to brain cells is likely to be a feature of brain ischemic tolerance. So far, GLUT1 and GLUT3 upregulation appears to be the primary changes for improving the transport of glucose at BBB and neuronal membrane level, respectively. However, it is likely that other molecules are also implicated, such as the Na+/D-glucose cotransporter (SGLT1) (105).

2. Excitatory amino acid transporters

Excitatory amino acid transporters (EAATs) in glia and neurons remove glutamate from the extracellular space, thereby contributing to terminate glutamate-mediated synaptic signaling and preventing extracellular glutamate to reach neurotoxic levels. Given the variety of mechanisms that can lead to excitotoxicity (85, 282), changes in EAATs density could be another adaptive change contributing to brain ischemic tolerance. However, both EAATs down- and upregulation may be considered potentially neuroprotective, depending on which aspect of ischemia-induced glutamate efflux is considered: 1) an upregulation of EAATs could be beneficial by increasing the efficiency of the removal of extracellular glutamate, or 2) a reduction of the EAATs density on the cellular membrane could reduce the amount of glutamate released by reversed uptake, reported as the most important route for glutamate efflux when ischemia is severe enough to produced anoxic depolarization (176, 343). In addition, both in vivo and in vitro studies dedicated to this issue have provided conflicting data so far.

Ischemic preconditioning produced by 10 min of MCAO in rats resulted in an upregulation of EAAT2 and EAAT3, but not of EAAT1 expression (322). In contrast, CSD preconditioning in the same species downregulated both the glial transporter isoforms EAAT1 and EAAT2 at 1, 3, and 7 days after preconditioning, with a maximal decrease at the 3-day time point, i.e., a time frame that coincides with ischemic tolerance (96). CSD preconditioning also reduced by two-thirds the cortical efflux of glutamate associated with the subsequent 200 min of focal ischemia, but one cannot ascertain from such studies whether this effect was effectively due to a decreased density of glutamate transporters, and thereby a reduction in glutamate leaking through reversed transport, or a consequence of reduced ischemic brain damage (96). Still in vivo, hypoxic preconditioning (i.e., 3-h exposure to 8% O2) of newborn rats 24 h before their exposure to severe hypoxia-ischemia protected all the brain regions that were assessed morphologically (i.e., cortex, striatum, and hippocampus); in contrast, EAAT1 proteins did not change in any region 24 h after preconditioning, whereas EAAT2 levels increased in the cortex by 55% but did not change in the hippocampus and were decreased by 48% in the striatum, suggesting that EAAT changes were not related to ischemic tolerance in this study (74).

When rat cortical cultures (primary neuronal) were exposed to sublethal oxygen-glucose deprivation (i.e., in vitro ischemic preconditioning), ischemic tolerance was associated with upregulation of EAAT2 and EAAT3 (whereas EAAT1 remained unaltered), and glutamate uptake was more efficient in the preconditioned cultures (338). However, with neuron/astrocyte cocultures, another group of investigators reported that a downregulation of the EAAT2 glutamate was associated with ischemic tolerance induced by ischemic preconditioning (210, 442).

Overall, these studies do not support the notion that altered EAATs expression is an essential and/or common contributor to brain ischemic tolerance.

C. Defense Mechanisms Against Oxidative Stress

A large body of evidence indicates that ROS contribute to the pathogenesis of brain damage produced by ischemia/reperfusion. ROS may originate from mitochondria in neurons, glia, and endothelial cells (for reviews, see Refs. 60, 256) or intravascularly from activated leukocytes and platelets (90). During recirculation, excessive NO formation may also contribute to ischemic brain injury through the formation of reactive nitrogen species such as peroxynitrite (i.e., reaction product of nitric oxide with superoxide radicals) (196). There is evidence suggesting that superoxide anion generation during preconditioning induced by transient ischemia is necessary for...
the subsequent development of ischemic tolerance (see Ref. 126 for recent in vitro data and Ref. 309 for review). For example, intravenous administration of human recombinant Cu/Zn-superoxide dismutase (SOD) to rats prior to ischemic preconditioning suppressed the development of tolerance against a subsequent MCAO insult, as well as the expression of HSP70 (255). Similarly, there is good evidence of protective and restorative properties for nitric oxide (NO), and these include the induction of gene expression programs that underlie brain preconditioning (71, 169, 196).

Against this background, it is logical to hypothesize that ischemic tolerance may involve an enhancement of defense mechanisms against ROS, and especially an upregulation of antioxidant enzyme activities (i.e., SODs, catalase, and glutathione peroxidase). Some data support this concept. In rats previously subjected to focal ischemic preconditioning, there was a significant reduction in the volume of the cortical lesion produced by a subsequent MCAO, and this ischemic tolerance was associated with a significant reduction of lipid peroxidation (69). In cultured neurons subjected to hypoxic preconditioning, the activity of the antioxidant enzymes glutathione peroxidase, glutathione reductase, and Mn-SOD were significantly increased, and superoxide and hydrogen peroxide concentrations following a subsequent episode of oxygen-glucose deprivation were lower in preconditioned cultures (14). However, according to the numerous studies dedicated to the key antioxidant enzymes SODs, catalase, and glutathione peroxidase, brain tolerance is not always associated with increased activities or upregulation of these enzymes. Whether or not these antioxidant enzymes are upregulated appears to depend, at least in part, on the preconditioning stimulus that was used.

1. SODs (Mn-SOD and Cu/Zn-SOD)

With hypoxic preconditioning, three separate studies using different experimental strategies all suggested that hypoxic tolerance, when present, was related to increased expression and activities of SODs (133, 143, 292). In contrast, ischemic preconditioning in rats induced sustained increased levels of SODs when induced by 5 min of four-vessel occlusion (86), but not when induced by 3-min MCAO (325). When brain preconditioning was induced in gerbils by an oxidative stress (287) or in rats by LPS (41), SOD brain activity increased with ischemic tolerance. Finally, CSD is a robust stimulus for brain preconditioning, but two separate studies with rats clearly suggested that CSD-induced ischemic tolerance was not mediated through the upregulation of SODs (253, 433).

2. Catalase

In rats, when 5 min of four-vessel occlusion was used to induce brain preconditioning, catalase activity increased significantly at all time points considered (5, 24, and 48 h postpreconditioning) with a peak at the 24 h time (86), but such a change was not observed with LPS-induced preconditioning (41). In vitro, when primary cultures of rat cortical neurons were subjected to hypoxic preconditioning, there was no subsequent increase in catalase activity (14).

3. Glutathione peroxidase

In rats, brain preconditioning induced by 3-min MCAO significantly reduced the cerebral infarct produced by 1-h MCAO within the 24- to 72-h postpreconditioning time window, but ischemic tolerance was not associated with any changes in the activities of glutathione peroxidase, nor in its neuronal expression (325). In vitro, when primary cultures of rat cortical neurons were subjected to hypoxic preconditioning, glutathione peroxidase and glutathione reductase activities were increased by 54 and 73%, respectively (14).

Whenever it is present, Cu/Zn-SOD (SOD1, cytosolic) and/or Mn-SOD (SOD2, mitochondrial) upregulation presumably contributes to brain tolerance because their overexpression, by itself, was neuroprotective in various models.

4. SOD1

Overexpression of this enzyme in transgenic mice was cerebroprotective during reperfusion following focal ischemia (125, 208), but not when MCAO was permanent (61); it reduced neuronal cell loss at 3 days after 8 min of cardiac arrest in some brain regions (207), which agrees with findings obtained with SOD1 overexpression in rats (62). It attenuated the neurotoxicity induced by the systemic administration of the mitochondrial toxin 3-nitropropionic acid (26); it protected cultured astrocytes exposed to oxygen-glucose deprivation for 5 h, and this beneficial effect was associated with the maintenance of glutathione at higher levels (428).

5. SOD2

Membrane lipid peroxidation, protein nitration (reflecting damage by peroxynitrite), and neuronal death, measured 1 day after 1 h of MCAO, were all significantly reduced in transgenic mice overexpressing human SOD2 (192). In contrast, apoptosis subsequent to transient focal cerebral ischemia was more pronounced in heterozygous SOD2 +/- knockout mice as indicated by enhanced accumulation of cytosolic cytochrome c and increased DNA laddering after ischemia (124).

The latter data (124) and other studies (125, 351, 398) suggest that the neuroprotective effect of SOD overexpression may be linked, at least partly, to an attenuation...
of the mitochondrial signaling of apoptosis to which ROS contribute.

In addition to the well-known antioxidant enzymes considered above, the thioredoxin system also contributes to cellular redox balance. Thioredoxins (Trxs) are small, ubiquitous proteins with two redox-active cysteine residues that participate in redox reactions through the reversible oxidation of their active center dithiol to a disulfide. Oxidized Trxs are reduced to the dithiol form by the thioredoxin reductase (TrxR) with the use of electrons from NADPH. Trxs and TrxR constitute the thioredoxin system (180). Mammalian Trxs include cytosolic thioredoxin-1 (Trx-1), mitochondrial thioredoxin-2 (Trx-2), a larger Trx-like protein p32TrxL, and other family members. The thioredoxin system is present in various CNS regions and emerges as an important contributor to the cellular defense against ROS (180). Furthermore, recent studies indicate that specific Trxs are also key regulators of fundamental cellular processes (e.g., gene expression, apoptosis, cell growth) through their interactions with various proteins such as redox-regulated kinase and transcription factors (303).

In contrast to SODs, catalase, and glutathione peroxidase, Trx upregulation appears to be consistently induced by preconditioning stimuli. Exposure of the rat retina to transient ischemia or hydrogen peroxide-induced oxidative stress induced mitochondrial Trx (135). In cultured human neuroblastoma cells, preconditioning induced by 2-h serum deprivation upregulated Trx and made the cells more tolerant to oxidative stress (9); through subsequent studies, the same group showed that the increased tolerance associated with upregulated Trx was blocked by Trx mRNA antisense nucleotide and a TrxR inhibitor (70) and that Trx upregulation may mediate the secondary induction of Mn-SOD and of the anti-apoptotic protein Bcl-2 (11). Hypobaric hypoxia-induced preconditioning increased Trx-2 mRNA expression in the rat brain (355); the same procedure also markedly strengthened the increased levels of Trx-1 and Trx-2 produced by a subsequent, severe hypobaric hypoxia (395, 396). It is also pertinent to mention that in rats subjected to MCAO, both Trx mRNA expression and protein levels rapidly decreased in the ischemic core (i.e., striatum and frontoparietal cortex), but increased in perifocal regions throughout the 4- to 24-h period after MCAO (403).

Trx upregulation may well contribute to brain tolerance, as procedures aiming to increase Trx levels were neuroprotective both in vivo and in vitro. Overexpression of Trx in transgenic mice attenuated focal ischemic brain damage and the associated neurological deficit, and these beneficial effects correlated with reduced cellular protein oxidation (402). The same mice were also more resistant to hippocampal damage produced by administration of the excitotoxin kainic acid (401). In mice, intravenous infusion of recombinant human Trx for 2 h after 90 min of MCAO reduced infarct volume, neurological deficit, and protein carbonyl content (i.e., a marker of protein oxidation) (153). Overexpression of Trx in PC12 cells prolonged their survival in culture medium lacking nerve growth factor (NGF) and serum, suggesting that Trx can delay neuronal apoptosis (170); subsequent studies showed that Trx was a direct inhibitor of the apoptosis signal-regulating kinase (ASK) 1 (352). In support of these data, Trx in the submicromolar range blocked mitochondria-mediated apoptosis in cultured human neuroblastoma cells (10). Finally, overexpression of p32TrxL protected cultured HEK-293 cells against glucose deprivation (180).

To conclude this section, it is likely that an enhancement of the cellular defense mechanisms against oxidative stress contributes to brain tolerance. Upregulation of the Trx system appears as a common feature, whereas upregulation of SOD, catalase, and glutathione peroxidase may only occur with some preconditioning stimuli. It is relevant to mention that Trx upregulation may be induced pharmacologically, as the monoamine oxidase type B (MAO-B) inhibitor deprenyl upregulated Trx in cultured neuroblastoma cells, and the associated neuroprotection was blocked by a Trx-mRNA antisense nucleotide (11).

D. Heat Shock Proteins

Heat shock proteins (HSPs) or stress proteins are found in all living cells, where they act as molecular chaperones, carrying out multiple functions that are essential to protein housekeeping. In normal cells, HSPs assist in the folding of newly synthesized proteins. Briefly, this is accomplished by HSPs reversibly and cyclically binding to exposed hydrophobic stretches of nascent polypeptides exiting ribosomes during translation. HSPs are also involved in the assembly and maintenance of multiprotein complexes, intraorganellar protein trafficking, and degradation of misfolded polypeptides (120). This section is focused primarily on inducible 72-kDa HSPs (HSP70), as these are well-characterized and the most studied with regard to cellular stress response and preconditioning.

1. Increased expression of HSPs with cellular stress, including brain preconditioning

Increased expression of HSP genes is a universal feature of the cellular response to insults, and it is now acknowledged that their chaperone activities contribute to cytoprotection during periods of stress (114). As proteins denature, HSPs bind to exposed hydrophobic stretches to stabilize the proteins; they also bind and attempt to disassemble denatured polypeptides that aggregate nonspecifically.
Many in vivo studies have shown that brain preconditioning is also associated with induction of the nonconstitutive HSP70 and other chaperones (66, 84, 138, 149, 171, 202, 278, 279, 372). For example, HSP70 was increased in the hippocampal CA1 region when gerbils were subjected to 2 min of global ischemia, a brief insult that markedly enhanced the survival of hippocampal neurons to a subsequent 5-min ischemia (202). The possibility of a temporal relationship between HSP70 upregulation and increased tolerance to ischemia was examined in several of these studies (66, 84, 278, 279). Chen et al. (66) reported that brief sublethal periods of global ischemia increased the tolerance to a subsequent permanent focal ischemia in rats and that HSP70 protein expression increased during times when tolerance was present (2–5 days post-preconditioning). The findings of Liu et al. (230) and Nishi et al. (278) also suggested a good correlation between increased HSP70 and ischemic tolerance. However, HSP70 also increased 1 day after preconditioning when tolerance was not yet present (66), and two other studies suggested that ischemic tolerance may not correlate well with HSP70 upregulation (84, 279). The latter data supported the notion that HSP70 (and other chaperones) are not solely responsible for ischemic tolerance and that they may not be required for cerebroprotection. This apparent discrepancy between these two sets of studies may be due to the fact that preconditioning increases HSP70 expression over a relatively long period of time, starting early after exposure to the initial stressor. This makes it possible for HSP70 to be also an early contributor to the signaling cascade(s) that leads to the development of ischemic tolerance. Indeed, there is evidence that HSP70 can regulate the activation of the nuclear factor κB (NFκB), a well-established “transducer” of preconditioning (94). In cell lines derived from immune cells, the double-stranded RNA-dependent protein kinase (PKR), known to be involved in the activation of NFκB, was modulated by HSP70 in a way that suggested a pro-survival function of HSP70 through activation of a PKR/NFκB-dependent protective pathway (115).

A convincing body of evidence indicates that HSP70 is required for adaptive cytoprotection and that high levels of HSP70 are neuroprotective.

2. Effect of suppression of HSPs

Early in vitro studies already suggested that HSP70 inactivation impairs adaptive cytoprotection. For example, microinjection of HSP70 antibodies into fibroblasts (335) and competitive inhibition of HSP70 gene expression in hamster ovary cells (183) markedly increased the vulnerability of these cells to thermal stress. Nakata et al. (271) applied the same experimental strategy to brain preconditioning and showed that the continuous infusion on the lateral ventricle of gerbils of HSP70 antibody or quercetin (i.e., an inhibitor of HSP70 expression), started whether 2 h before or 3 h after the preconditioning ischemia, prevented the induction of ischemic tolerance in the CA1 hippocampus.

3. Effect of overexpression of HSPs

Reciprocally, a large body of evidence indicates that the overexpression of HSP70 and other HSPs is neuroprotective, both in vitro and in vivo (163, 194, 195, 233, 242, 319, 326, 330, 448). Virus-mediated overexpression of HSP70 improved the survival of striatal neurons when rats were subjected to 1 h of MCAO, and the survival of hippocampal neurons after systemic kainic acid administration (448). The same procedure protected hippocampal neurons in rats subjected to global cerebral ischemia (195). Neuronal damage produced by 25 min of bilateral carotid occlusion was reduced in transgenic mice over-expressing HSP70 compared with their wild-type littermates (194). In addition, primary hippocampal cultures prepared from these transgenic mice were more resistant to toxic levels of glutamate and oxidative stress. For example, exposure to 10 μM free iron produced a 26% increase in lactate dehydrogenase release from neurons cultured from wild-type mice, but a 7% increase in neurons cultured from HSP70 transgenic mice (194). In transgenic mice overexpressing rat HSP70, with HSP70 primarily expressed in neurons under normal conditions, cerebral infarction was markedly reduced after both 6 and 24 h of permanent MCAO (330). Subsequent studies showed that the same mice had an increased brain tolerance to neonatal hypoxia/ischemia (242). HDJ-2 is a member of the HSP40 family that promotes protein folding both by binding to unfolded proteins and by regulating the activity of HSP70. Virus-mediated HDJ-2 overexpression in cultures of primary mouse astrocytes markedly reduced cell death produced by 24 h of glucose deprivation or 8 h of oxygen-glucose deprivation (326). Geldanamycin is a benzoquinone ansamycin that binds HSP90, releases heat shock factor 1 (HSF1), and stimulates HSP gene transcription. When injected into the lateral cerebral ventricles of rats prior to 2 h of MCAO, geldanamycin decreased the infarct volumes by 55.7% and the number of TUNEL-positive cells by 30% in the cerebral cortex. Western blots showed that this protection was associated with an 8.2- and 2.7-fold increase in HSP70 and HSP25 protein, respectively (233).

It is clear from all these data that HSP70 overexpression is cerebroprotective when induced prior to ischemia onset, but one study suggested that it may be also effective when instigated after the insult. When viral vectors were used to overexpress HSP70 in the striata of rats at 0.5, 2, and 5 h after the onset of 1-h MCAO, significant neuroprotection was observed at the 0.5 and 2 h time points, but not at 5 h (163).
4. Cytoprotective mechanisms of HSPs

In line with the essential functions of HSPs in cellular protein management, the protective effects of HDJ-2 or HSP70 overexpression were associated with a reduction of the protein aggregation resulting from ischemic injury (137, 326, 400) (see Fig. 1). However, neither the ability of HSP70 to fold denatured proteins nor its interactions with cochaperones or other proteins that bind to the NH$_2$-terminal half of HSP70 may be essential to ischemic protection. This was suggested by studies with two mutated HSP70s, both capable of binding to denatured proteins and maintaining their solubility, but no longer able to fold proteins (i.e., folding deficient mutants); the overexpression of either of these two HSP70 mutants still protected the brain of rats subjected to 2 h of MCAO followed by 24 h of reperfusion and cultured astrocytes from oxygen-glucose deprivation (400). These protective actions were also associated with a better maintenance of mitochondrial physiology (i.e., inhibition of mitochondrial membrane potential change, better maintenance of state IV respiration, and reduced generation of reactive oxygen species) in astrocytes subjected to glucose deprivation (299). Mitochondria play a central role in the induction of two different types of apoptosis (Fig. 2): “intrinsic” caspase-dependent apoptosis, through its release of the proapoptotic factor cytochrome c, and caspase-independent apoptosis, through its release of apoptosis-inducing factor (AIF) (53). Accordingly, HSP70-induced mitochondrial protection should be expected to promote cell survival after an ischemic insult, and such an anti-apoptotic action is supported by the demonstration that overexpression of HSP70 reduced the release of cytochrome c in the cytosol (242) and the nuclear translocation of AIF (242, 400).

In addition, HSP70 has the capability to antagonize apoptosis directly. With regard to intrinsic, caspase-dependent apoptosis, HSP70 inhibits events leading up to mitochondrial membrane permeabilization and cytochrome c release, primarily by inhibiting the translocation of Bax (a proapoptotic member of the Bcl-2 family) to mitochondria, apparently through its ability to suppress JNK activation (382) (Fig. 2). Preconditioning is known to be associated with an upregulation of the antiapoptotic oncogene Bcl-2 (see sect. II E), and HSP70 overexpression resulted in a marked upregulation of Bcl-2 in both injured and uninjured neurons (195). However, the latter study did not ascertain whether this effect was due to a direct action of HSP70 on Bcl-2 expression. Finally, HSP70 was reported to bind apoptosis protease activating factor-1 (Apaf-1), thereby preventing the constitution of the apoptosisosome (i.e., the Apaf-1/cytochrome c/caspase-9 activation complex) (29, 354) (Fig. 2). However, this action was subsequently challenged by Steel et al. (384) who proposed that the key action of HSP70 with regard to intrinsic apoptosis is inhibition of cytochrome c release, either by acting directly on the mitochondria or at some stage upstream. Clearly, Apaf-1 is not the only target for HSP70 antiapoptotic action, because HSP70 still protected Apaf-1—/— cells against apoptotic death (331), and overexpression of mutated HSP70 lacking the ATP-binding domain, which is required for Apaf-1 binding, still protected astrocytes from ischemic injury (400). With regard to caspase-independent apoptosis, HSP70 neutralizes the mitochondrial effector AIF in a reaction that appears to be independent of the HSP70 ATP-binding domain (331). Interestingly, distinct HSP70 domains are involved in the prevention of mitochondrial AIF release and the sequestration of AIF in the cytosol, which supports the notion

FIG. 1. Diagram illustrating the multiple actions of 70-kDa heat shock protein (HSP70) that may contribute to ischemic tolerance. The dotted arrows indicate potential interactions between different HSP70 actions. Although protein folding is a key function of HSP70, it may not be essential to increased tolerance as the overexpression of mutated, folding-deficient HSP70 was still cerebroprotective (400). *HSP70 effects that did not depend on its protein folding capability (290, 346, 400, 440). Note that HSP70 may also contribute to the initiation of the inflammatory response (i.e., at an early stage after the induction of preconditioning) by acting as a “danger signal” for the toll-like receptors (TLR2/4) (see Fig. 3). MMPs, matrix metalloproteinases; BBB, blood-brain barrier.

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that HSP70 has the potential to suppress apoptotic cell death through various mechanisms (346).

In contrast to apoptosis, which is a highly coordinated process subject to complex regulation, necrosis is generally considered to be an unregulated event that occurs whenever cells are damaged beyond their repair capability. However, emerging evidence suggests that cells could still die via necrosis following a moderate stress that did not produce irreparable cellular damages. Furthermore, signaling pathways such as death receptors, kinase cascades, and mitochondria participate in both apoptosis and “programmed” necrosis (324). Within this context, it is relevant to note that HSP70 overexpression in cultured astrocytes, whether produced by retroviral transfection or pharmacological induction, reduced glucose deprivation-induced necrosis (438, 439). This antinecrotic effect of HSP70 may well be linked to its action on intrinsic apoptosis signaling, upstream of mitochondria (Fig. 2), as HSP70 (whether wild-type or folding deficient mutant) inhibited JNK-related steps of necrotic pathway induced by transient energy deprivation in H9c2 myogenic cells (128, 440).

There is also evidence that HSP70 may suppress important effectors of the postischemic inflammatory response, the matrix metalloproteinases (MMPs), proteolytic enzymes that function in the extracellular matrix to degrade its main components, i.e., collagens, laminin, and proteoglycans. Ischemic insults elicit a robust inflamma-
tory reaction to which MMPs contribute by promoting increased vascular permeability, BBB disruption, and inflammatory cell influx (75). Several lines of evidence suggest that MMPs, and especially MMP-9 (gelatinase B), contribute to the genesis of ischemic lesions. 1) Higher levels and proteolytic activities of MMP-2 (gelatinase A) and MMP-9 were demonstrated in ischemic lesions produced by MCAO in rats (317, 340), and in human brain tissue after ischemic and hemorrhagic stroke (339). 2) The temporal profile and distribution of these changes suggested that MMP-9 may contribute to secondary tissue damage and vasogenic edema, whereas MMP-2 may be involved in tissue repair at a later stage (123, 340). 3) Inhibition of MMP-9, whether induced by antibody neutralization, drugs, or genetic manipulations, had protective effects in stroke models (15, 134, 147, 337). In contrast to single, severe ischemic insults, preconditioning stimuli appear to suppress MMPs. 1) Ischemic preconditioning in rats reduced the high expression of MMP-9 that was associated with subsequent MCAO, and this effect correlated with decreased edema and BBB disruption (454). 2) Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is a specific, endogenous inhibitor for a group of MMPs, among which MMP-9 is the preferred target, and ischemic preconditioning induced its expression at a time when ischemic tolerance is increased (431). Finally, HSP70 upregulation with preconditioning may contribute to suppress ischemia-induced MMP upregulation, as virus-mediated HSP70 overexpression in cultured astrocytes suppressed MMP-2 and MMP-9 (220). Unexpectedly, there is evidence suggesting that extracellular HSPs may be released from cells under stress, to act as potential danger signals to immune effector cells (see sect. iv A).

From this section, HSP70 clearly emerges as a molecular chaperone with multiple cytoprotective functions that make it a relevant therapeutic target (380, 460).

E. Control of Cell Death/Survival

Necrosis is generally considered to be the main cause of cell death in severely ischemic brain regions; in this case, the death is rapid and the extent of tissue damage (infarct volume) is closely related to the severity and duration of the shortage in blood supply (225, 249). In contrast, at the periphery of the ischemic core with focal insults, or in selectively vulnerable regions such as the hippocampus CA1 after a short period of ischemia, neurons may die over a longer period with morphological signs and biological features indicative of apoptosis (67, 112). In this section, emphasis is placed on the well-documented notion that an effective suppression of apoptosis is associated with brain tolerance. However, it is important to stress that possible changes in necrosis are not ruled out 1) because the smaller infarcts observed with ischemia-tolerant brains imply reduced necrosis, though this reduction is likely to be a consequence rather than a cause of increased tolerance; and 2) because more recent data indicate that the classical necrosis/apoptosis subdivision is an oversimplification of the multiple and more diverse mechanisms of cell death that are involved in ischemic brain damage. Indeed, depending on the insult type and intensity, and on the cells bioenergetic state, necrosis together with different forms of apoptosis and other types of programmed cell death may occur and shape distinct morphological changes (221). In particular, secondary necrosis may develop as an abortive form of apoptosis, subsequent to a switch from apoptotic cell death to necrotic cell death triggered by intracellular ATP depletion (277).

Classical (i.e., caspase-dependent) apoptosis can be initiated by two major pathways: the “intrinsic” mitochondrial pathway and the “extrinsic” death receptor pathway, both converging on a family of caspses (211). In the “intrinsic” pathway, the release of mitochondrial cytochrome c initiates the activation of the caspase cascade via the constitution of the apoptosome The (Apaf-1/cytochrome c/caspase-9 complex). Assembly of the apoptosome allows pro-caspase-9 to be autoactivated, and this is followed by the recruitment and activation of pro-caspase-3. Cleaved caspase-9 remains bound to the apoptosome, which recruits and activates executioner caspses such as caspase-3, -6, and -7. Caspase-3 cleaves the inhibitor of caspase-activated deoxyribonuclease (DNase), thereby activating DNase, resulting in internucleosomal DNA fragmentation (211). Alternatively, the extrinsic pathway is driven by activation of plasma membrane death receptors and activation of caspase-8 (note that the extrinsic pathway has been omitted from Fig. 2). Both pathways converge on caspase-3, and cross-talk between pathways has been described. Mitochondrial, caspase-independent apoptosis is initiated by the release of AIF from the intermembrane mitochondrial space to the cytosol, from which it translocates to the nucleus. As AIF by itself has no endonuclease activity, the resultant DNA-degrading capacity requires the recruitment of downstream nucleases such as cyclophilin A (211).

The central role of mitochondria in intrinsic caspase-dependent apoptosis and caspase-independent apoptosis, and the convincing evidence that mitochondria preservation is associated with ischemic tolerance (see sect. iv A), already indicates that apoptosis may be suppressed in the tolerant brain, and this has been demonstrated by numerous studies (54, 316, 405, 464). This notion is further strengthened by the multiple antiapoptotic actions of HSP70 (see sect. iv D and Fig. 2). To avoid repetition, only complementary evidence of apoptosis inhibition in the ischemia-tolerant brain is provided next.

The p53 tumor suppressor gene is as an important transcription factor that promotes apoptosis following
neuronal injury (81), and its activation appears to be reduced in the ischemia-tolerant brain. 1) Both p53 mRNA and protein levels increased in the rat hippocampus 24 h after transient, forebrain ischemia, but this change was markedly diminished when ischemic preconditioning was induced 48 h prior to the test ischemia (412). 2) The finding that the mKATP channel agonist diazoxide inhibited the apoptosis of cultured hippocampal CA1 neurons exposed to hypoxic/anoxic conditions was already mentioned (see sect. 1A). This neuroprotective effect was associated with a reduced expression of p53 (168).

Several in vivo and in vitro studies carried out in rats, mice, or gerbils, and using various stimulus for preconditioning and different subsequent test insults, have been dedicated to changes in the Bcl family proteins (i.e., Bcl-2, Bcl-xL, and Bax) that play a pivotal role in the control of cell death. Overall, the data from these studies suggest strongly that ischemic tolerance is associated with a shift of Bax/Bcl-2 Bcl-xL ratio to suppress apoptosis. Findings supporting this notion include the following: 1) Persistent overexpression of the antiapoptotic Bcl-2 and Bcl-xL genes (44, 190, 246, 248, 349, 366, 369, 434, 436); 2) the proapoptotic Bax gene upregulation that occurred after the test insult was reduced in the preconditioned brain of rats (44, 349); Bax expression, however, remained unchanged in a study with mice (434); and 3) cerebroventricular infusion of Bcl-2 antisense oligonucleotide reduced the expression of Bcl-2 and suppressed ischemic tolerance in the rat brain (369).

Although most of the anti-apoptotic changes reported so far in the tolerant brain are related to events that occur upstream of caspase-3 activation (i.e., preceding the execution step of the intrinsic, caspase-dependent apoptosis; Fig. 2), some data also suggested that preconditioning may promote neuronal survival despite caspase-3 activation. Preconditioning of rat cortical cultures by a sublethal exposure to cyanide, or in vivo by 10-min MCAO, produced significant caspase-3 cleavage (i.e., activation) but without subsequent cell death (246). This study also suggested that some degree of caspase-3 activation was required for the ultimate development of ischemic tolerance, at least with the models and experimental procedures involved (246). In agreement with these data, in rats subjected to transient global ischemia by four-vessel occlusion for both preconditioning and test insult, the subsequent 10-min ischemia activated caspase-3 but failed to increase nuclear CAD, induce p75NTR, and cause DNA fragmentation (i.e., downstream events toward execution of cell death) (405). Other data from this study suggested that preconditioning, by preserving mitochondria, may reduce the release of "second mitochondria-derived activator of caspases/direct IAP-binding protein with low pi" (Smac/DIABLO), and thereby preserve the capacity of cellular inhibitor-of-apoptosis protein (cIAP) to directly inhibit activated caspase-3 (405).

The protooncogene Akt is a central component of the phosphatidylinositol 3-kinase (PI 3-kinase) signal transduction pathway. Downstream effector of PI 3-kinase, Akt promotes cell survival by phosphorylating (i.e., inactivating) several proapoptotic proteins, including caspase-9, Bad (a Bcl family protein), forkhead transcription factors (FKHR), and glycogen synthase kinase 3 (GSK3) (390). Several lines of evidence support the notion that Akt activation (i.e., maintenance of its phosphorylated form) is neuroprotective. 1) When apoptosis of primary cultured hippocampal neurons was induced by hypoxia or NO treatments, adenovirus-mediated overexpression of activated-Akt suppressed neuronal including (443); and 2) after 4 h of MCAO in mice, phosphorylated Akt was decreased in the ischemic core but increased in cortical peri-infarct region; double staining showed different cellular distributions for phosphorylated Akt and DNA fragmentation, and pretreatment with a PI 3-kinase inhibitor prevented the increased Akt phosphorylation and promoted DNA fragmentation (281). However, in vivo studies dedicated to the possibility that Akt activation may contribute to adaptive cytoprotection have provided inconsistent data. On the one hand, data from two studies support the notion that Akt activation may contribute to ischemic tolerance. 1) Following sublethal cerebral ischemia in gerbils, Akt phosphorylation was persistently stimulated in the hippocampal CA1 region and, in contrast to nonpreconditioned animals, Akt phosphorylation showed no obvious decrease after the subsequent lethal ischemia (447). In the same study, intracerebroventricular infusion of the PI 3-kinase inhibitor wortmannin prior to preconditioning blocked both the increase in Akt phosphorylation and the neuroprotective action of preconditioning (447). 2) Preconditioning, induced by transient focal ischemia in rats, reduced neuronal death produced by subsequent lethal MCAO in the penumbra, and this neuroprotective effect was associated with persistent Akt activation in this region (268). On the other hand, data from two other studies rather suggested that Akt may play a role in neuronal survival only after ischemia, as Akt phosphorylation occurred early after the insult and was not sustained (274, 365). Even more conflicting are the data from a recent study with rat PC12 cells (159). In this cell line, ischemic tolerance induced by exposure to 6 h of oxygen and glucose deprivation (OGD) was actually associated with reduced phosphorylation of Akt and of its targets GSK-3, FoxO4 (a FKHR family member), and murine double minute 2 (MDM2) protein, and decreasing the availability of phosphorylated Akt, either pharmacologically or by transflecting PC12 cells with inactive Akt constructs, reduced cell death produced by OGD and increased the protective effect of preconditioning (159). Finally, it is also relevant to mention that reduced Akt phosphorylation...
tion was also observed in bats and ground squirrels during hibernation, another type of adaptation that includes tolerance to pronounced reduction of cerebral perfusion (51, 103).

F. DNA Repair

Lipids and proteins have received the most attention in the field of oxidative damage and cerebral ischemia, but oxidatively damaged DNA is particularly detrimental to cells and, in contrast to altered lipids and proteins that can be removed and replaced as part of normal turnover, DNA lesions must be repaired. The pathways for the removal of DNA lesions can be divided into base excision repair (BER) and nucleotide excision repair (NER) (106). Quantitatively, BER is by far the most important route for the removal of the majority of oxidative lesions. At its simplest, BER is initiated by a specific enzyme that removes the oxidized base, e.g., 8-oxoguanine glycosylase (OGG1), leaving an apurinic-apyrimidinic (AP) site, that is subsequently removed by an AP lyase activity (a separate enzyme or glycosylase associated). Complementing BER, NER is directed principally towards bulky lesions, such as cyclobutane thymine dimers (TT). With both BER and NER, excision is followed by gap filling and ligation.

Oxidative DNA lesions are a feature of ischemia/reperfusion injury, independent from DNA fragmentation detected using TUNEL (83). For example, in gerbils subjected to 5 or 10 min of global ischemia, a marked accumulation of the oxidative DNA damage product 8-hydroxy-2’-deoxyguanosine (8-OH-dG) occurred as early as 15 min after recirculation in the hippocampus and persisted for several days (7, 17). Some data also suggested that oxidatively damaged DNA may contribute to lesion progression in stroke models. In rats subjected to permanent MCAO, 16–72 h after ischemia onset, 8-OH-dG levels and AP sites were selectively increased in the peri-infarct region, and their colocalization with DNA single-strand breaks and DNA fragmentation suggested a contribution to lesion progression (265).

Defective repair of oxidatively damaged DNA can be a potent initiator of neurodegeneration, and this is most evident in xeroderma pigmentosum (XP), an autosomal recessive disease arising from mutations in key genes associated with NER (106). Few studies have been dedicated to ischemia-induced alterations of DNA repair, but they concur in suggesting that these changes may play a role in the development of postischemic neuronal death. 1) In mice subjected to 1-h MCAO, the expression of Ku70 and Ku86 (multifunctional DNA repair proteins that bind to DNA strand breaks and triggers DNA repair) was decreased in ischemic brain tissue as early as 4 h after reperfusion and remained reduced until the 24-h time point (199); 2) MCAO for 100 min in rats increased mitochondrial BER activity for ~1 h, but a decline to less than control levels developed over the following 4–72 h (64); and 3) in rats subjected to transient global forebrain ischemia, the immunoreactivity of proliferating cell nuclear antigen (PCNA, required for NER) was largely abolished during reperfusion in the vulnerable CA1 neurons, prior to their death (411).

A correlation between increased tolerance to MCAO in rats and marked attenuation of DNA lesions was recently reported, and this may be a consequence of improved DNA repair since ischemic preconditioning induced a marked increase of β-polymerase-mediated BER activity that extended throughout the course of ischemic tolerance (224). This study agrees with previous findings: 1) ischemic preconditioning in rats induced the expression of the Ku 70 protein, with a time frame overlapping with ischemic tolerance (397); 2) 1 h of MCAO in rats markedly induced BER activity in the frontoparietal cortex (i.e., region that survived the insult) for at least 72 h (214); and 3) a 30-min, preconditioning MCAO in rats induced mild oxidative mitochondrial DNA (mtDNA) damage and activated key mitochondrial BER pathway enzymes for up to 72 h (64). The latter finding may be especially relevant, first because of the central role of mitochondria in several aspects of ischemic brain damage, and second because mtDNA is more exposed to oxidative damage than its nuclear counterpart due to the effective generation of ROS by mitochondria and mtDNA lacking the structural features that protect nuclear DNA. As a consequence, mtDNA damage is more extensive and persists longer than nuclear DNA damage (441). Altogether, these results suggest strongly that enhanced DNA repair constitutes an important endogenous mechanism to protect the brain against ischemia-induced DNA damage.

G. Self-Repair and Plasticity (Neurogenesis/Synaptogenesis and Growth Factors)

It is now established that, throughout the life of mammals, new neurons can be generated from resident progenitor/stem cells in the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles. The proliferation potential of these progenitor cells provides the adult brain with a capacity for plasticity and self-repair through neurogenesis, which responds to both external stimuli (e.g., environmental enrichment) and injury (e.g., ischemic and traumatic insults) (for reviews, see Refs. 226, 280). Whether this capacity for self-repair is activated in the tolerant brain was examined by Naylor et al. (276), using MCAO in rats for both preconditioning (10-min occlusion) and test insult (1-h occlusion) applied 3 days later. As in previous studies, progenitor cell proliferation increased after focal
ischemia alone (by 3.9-fold), but this occurred also after preconditioning alone (2.7-fold increase), i.e., in the absence of any infarction. Interestingly, in both ischemia and preconditioned groups, \( \sim 45\% \) of the progenitor cells that proliferated in week 1 survived to the end of week 3, and \( \sim 21\% \) of those matured into cells expressing a neuronal marker. Similarly, exposure of newborn rats to 5 min of anoxia did not produce any detectable cell death, but promoted cell proliferation in the subventricular zone and hippocampal dentate gyrus during the following 3 wk; these newly generated cells expressed neuronal markers, migrated to specific sites such as the CA1 layer, and neurogenesis was associated with improved memory at 40 days (321). In contrast, neurogenesis was not detected when ischemic tolerance in the hippocampus was induced by 2 min of bilateral carotid occlusion in gerbils (229).

Studies of growth and neurotrophic factors have provided another line of evidence supporting the notion that neurogenesis is activated in the tolerant brain. Several growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), IGF-I, brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) influence neurogenesis, both in the intact and ischemic brain (for review, see Ref. 226), and preconditioning upregulates these factors. 1) bFGF expression was increased in cultured rat cortical neurons 24 h after hypoxic preconditioning (353), in the rat cortex and hippocampus at 12 and 24 h after cortical spreading depression (244), and in the rat retina at 6- and 48-h after ischemic preconditioning (59). 2) Cerebral mRNA expression of both EGF and IGF-I was increased by preconditioning in the study of Naylor et al. (276) outlined previously. 3) BDNF was also upregulated for several days in the rat cortex after spreading depression (444) and by ischemic preconditioning in rats, albeit only at the early time points of 30 min and 6 h (414). 4) For VEGF, see section \( \nu \). A number of studies have also demonstrated that the neurogenesis associated with ischemic brain lesions can be enhanced by supplementing the ischemic brain with EGF, bFGF, IGF-I, or VEGF (19, 91, 399, 415); note the negative effect reported with BDNF (215).

However, the key issue of whether or not postsischemic neurogenesis is enhanced in the tolerant brain remains unresolved, because preconditioning had neither an inhibitor nor an additive effect on focal ischemia-induced progenitor cell proliferation in the comprehensive study of Naylor et al. (276).

Synaptogenesis (i.e., axonal sprouting and changes in dendritic spines) is another potential mechanism for plasticity and self-repair after an ischemic injury to the brain. In adult rats, poststroke axonal sprouting resulted in a substantial remapping of the connections of the somatosensory cortex adjacent to the infarct, with a unique profile of neuronal growth-promoting genes (57, 58). With regards to dendritic reorganization, changes preceding delayed neuronal death produced by transient ischemia in the rat hippocampal CA1 appear to be associated with neurodegeneration (238, 344), whereas delayed changes such as the increase in dendritic density in regions surrounding a cortical ischemic lesion may reflect morphological plasticity and repair (2). As posts ischemic synaptogenesis in rats could be enhanced by treatments initiated after the insult (e.g., environmental enrichment, \( \nu \)-amphetamine therapy) (46, 394), the possibility that preconditioning may promote these processes is another interesting issue that was addressed recently by Corbett et al. (77). In their experiments with gerbils, compared with appropriate controls, dendritic spine densities in hippocampal CA1 were significantly higher 3 days after preconditioning alone (i.e., 2 separate episodes of 1.5-min bilateral carotid occlusion, 24 h apart), and also 10 and 30 days after the 5-min test ischemia when it was preceded by preconditioning. Clearly, this topic deserves further attention.

III. SYNAPTIC TRANSMISSION IN THE TOLERANT BRAIN

A. Balance Between Glutamatergic and GABAergic Transmission

Excitotoxic glutamate receptor activation or malfunction is widely thought to contribute to ischemic brain damage, especially to delayed death of vulnerable hippocampal neurons after transient ischemia (for example, see Refs. 252, 295). GABAergic disinhibition may also contribute to lesion progression, because neuronal hyperexcitability associated with a sustained downregulation of \( \text{GABA}_A \) receptors was demonstrated in peri-infarct regions (327, 328, 356), although this response could as well promote plasticity and recovery from the ischemic injury (357). From this information, one could expect that the balance between glutamatergic excitation and GABAergic inhibition may be shifted in favor of the latter in tolerant brain, and this notion is supported by several findings related to GABAergic transmission: 1) Preconditioning induced by 2.5 min of global ischemia in gerbils resulted in a significant and sustained increase of \( [3H] \)-muscimol binding to \( \text{GABA}_A \) receptors (375). 2) In rats, ischemic preconditioning increased the activity of glutamate decarboxylase (i.e., the primary enzyme for GABA synthesis) and resulted in a more pronounced release of GABA during the subsequent 10-min global ischemia (87); however, such an effect of preconditioning on amino acid neurotransmitter release during the test ischemia was not found in a previous study with gerbils (272). 3) In rat brain cortical slices, preconditioning shifted the release of glutamate and GABA during the subsequent test hypoxia/
hypoglycemia in favor of inhibition (182). 4) Twenty-four hours after preconditioning by exposure to high-potassium medium, cultured cortical neurons exhibited an enhanced depolarization-induced presynaptic release of GABA that contrasted with a modest decrease in glutamate release (144).

With regard to scavenging of extracellular glutamate in the tolerant brain, the data reviewed in section II do not support the notion that altered EAAT expression is an essential feature of ischemic tolerance. However, an up-regulation of glutamine synthase was found after preconditioning with 3-nitropropionic acid, and as this enzyme converts glutamate to glutamine in astrocytes, this change was interpreted as a potential contributor to increased extracellular glutamate scavenging (166).

As transient, severe global cerebral ischemia selectively reduced the expression of GluR2 in CA1 hippocampal neurons, thereby promoting Ca\(^{2+}\) permeability of AMPA receptors and postischemic cell death (295, 306), several studies have examined whether changes in GluR2 expression are associated with ischemic tolerance. One day after ischemic preconditioning in rats, the expression of the AMPA receptor subunit GluR2 protein was actually increased in the CA1 region (206), but complementary experiments pointed to a selective upregulation of GluR2/GluR2\(^{+}\) flop in tolerant animals, and the latter change proposed as potential mechanism for enhanced AMPA receptor desensitization leading to ischemic tolerance (6). However, similar findings were not found in the gerbil hippocampus CA1 after ischemic preconditioning (376, 404), in the mouse cortex after preconditioning induced with spreading depression (63), nor in rat hippocampal neuronal cultures exposed to sublethal OGD (450).

There is convincing evidence that NMDA receptor activation is critical to the induction of ischemic tolerance in neurons (179, 379), but whether some NMDA receptor changes contribute to adaptive cytoprotection remains unclear. Two separate studies do not suggest that ischemic tolerance is associated with changes in the expression of NMDA receptor subunits (i.e., NR1, NR2A, and NR2B) (3, 63). However, changes in NMDA receptor function may occur. In hippocampal slices prepared from gerbils 2–3 days after ischemic preconditioning, anoxia-induced long-term potentiation (anoxia-LTP, known to be NMDA receptor mediated) was inhibited (191). One day after sublethal exposure of cultured cortical neurons to cyanide, whole cell recordings of NMDA-induced currents revealed a significant increase in voltage-dependent extracellular Mg\(^{2+}\) block that could decrease the overall current flowing through the channel, but the block reverted to control levels within 48 h (3).

Finally, with regard to metabotropic glutamate receptors (mGluR), only mGluR1b and mGluR5 protein levels were transiently downregulated in the preconditioned hippocampal CA1 of gerbils, but this occurred 8 h after induction of preconditioning by 2.5 min global ischemia, i.e., prior to the window of delayed ischemic tolerance (377).

Overall, it appears that changes in GABAergic transmission, both pre- and postsynaptic, are more likely to contribute to a shift of the glutamate/GABA balance toward inhibition in the tolerant brain.

B. Role of Adenosine

Adenosine and A1 receptors play a key role in the induction of brain tolerance (37, 158), but whether adenosine contributes also to the subsequent, delayed adaptive cytoprotection is less clear. In rats, ischemic preconditioning increased A1 receptor immunoreactivity in the hippocampal CA1 at days 1, 3, and 7 after preconditioning induction, i.e., within the window of ischemic tolerance (461), but such a change was not found in mice after sublethal 3-nitropropionate treatment (427), and whether this increase of A1 protein level was associated with functional gain remains to be confirmed. As A1 activation mainly decreases excitatory synaptic transmission in the CNS (100), should A1 receptors be upregulated in the tolerant brain, then adenosine either released as neuromodulator or as ATP breakdown product of ATP may contribute with GABAergic changes to shift the excitatory/inhibitory balance towards inhibition (see sect. IV). Within this context, it is relevant to mention that, without any preconditioning, endogenous activation of A1 receptors contributed to the suppression of synaptic activity occurring at the onset of global ischemia in both hippocampus CA1 and cortex, indicating that A1 receptor-mediated “shut down” of synaptic activity with deficient energy supply is already effective in the naive brain (113, 174).

C. Nicotinic-Cholinergic Transmission

In addition to epidemiological and clinical studies suggesting that chronic exposure to nicotine may be cytoprotective, several lines of evidence indicate that activation of nicotinic acetylcholine receptors (nAChR) can provide neuroprotection both in cell culture systems and animal models (for review, see Ref. 293). 1) Nicotine protected primary cortical cultures from hypoxia-induced apoptosis and glutamate excitotoxicity, and these effects were mediated by both \(\alpha\)7- and \(\beta\)2-containing nAChR subtypes (157, 388). 2) The selective partial \(\alpha\)7-agonist 3-(2,4-dimethoxybenzylidene)-anabaseine dihydrochloride (GTS-21) protected hippocampal CA1 neurons against delayed cell death produced by transient global brain ischemia in gerbils (275); the acetylcholinesterase inhibitor donepezil reduced the infarct volume produced by MCAO in rats, and this effect was prevented by the nAChR antagonist mecamylamine (122).
With regard to preconditioning, 1 day after repeated CSD in mice, robust ischemic tolerance was associated with a very marked and selective increase in α7-nAChR protein levels (63). Although it remains to be verified that an α7-nAChR functional gain resulted from the increased α7-subtype protein level, this discovery is worth discussing. The upregulation of α7-nAChR in the tolerant brain could be neuroprotective through direct activation of processes at neuronal level, because prolonged exposure of cultured neurons to nicotine was neuroprotective, and this treatment upregulated α7-nAChR (186), and α7-nAChR overexpression in PC12 cells, by itself (i.e., without nicotine), reduced the effects of hypoxia on cell cycle (G1 arrest) and DNA fragmentation (420). In addition, in the brain parenchyma, preconditioning-induced α7-nAChR upregulation could contribute to reduce the brain inflammatory responsiveness that underlies ischemic tolerance (Fig. 3). Indeed, the existence of a brain cholinergic anti-inflammatory pathway mediated by α7-nAChR is now well substantiated: macrophages, microvascular endothelial cells, and microglia express α7-nAChR on their surface, and the activation of these receptors resulted in immunosuppressive effects (350, 370, 451).

D. Other Changes in Ion Channels, Exocytotic Mechanisms, and Receptors

The previous sections (i.e., sect. III, A–C) discuss possible, inherent cerebroprotective strategies that depend on changes in synaptic transmission. These were selected, essentially, on the grounds of data availability. Given the complexity of synaptic transmission (exocytosis mechanisms, receptors, synaptic modulation, synaptic plasticity, etc.), it is clear that numerous studies are still required to provide a clear picture of the changes in synaptic machinery that are associated with, and contribute to, ischemic tolerance. Next, and again on the grounds of data availability, attention is given to K<sub>ATP</sub> channels and cannabinoid receptors.

Beside the possible contribution of mK<sub>ATP</sub> channels to ischemic tolerance (see sect. II A), that of cell surface membrane K<sub>ATP</sub> channels deserves to be mentioned here because these ATP-operated channels appear as important effectors for the coupling between energy availability and neuronal excitability. There is supporting evidence that K<sub>ATP</sub> channels play a protective role against energy deficiency, already in the naive brain (for review, see Ref. 21); for example, cell surface K<sub>ATP</sub> channels mediated the hyperpolarization that precedes anoxic depolarization in hippocampal slices (257). A number of early studies also suggested that surface K<sub>ATP</sub> channels are essential for the induction of preconditioning and subsequent ischemic tolerance (37, 158, 308, 334). However, more recent data, interpreted with an improved knowledge of mK<sub>ATP</sub> channels and K<sub>ATP</sub> channel drug selectivity (i.e., effects on mitochondrial versus surface K<sub>ATP</sub> channels; Ref. 132) have led to the question of the role of surface K<sub>ATP</sub> channels in ischemic tolerance. For example, a study with

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**FIG. 3.** Inflammatory responses during preconditioning and ischemic tolerance. This diagram illustrates the concept that preconditioning produces a mild inflammatory response subsequent to Toll-like receptor (TLR) activation, which results in the production of several negative-feedback inhibitors of inflammation, ultimately leading to reduced inflammatory responsiveness in the tolerant brain (see text and Ref. 188 for details). TLRs are transmembrane receptors, present in microglia and astrocytes, acting as pathogen sensors. However, some TLRs respond also to molecules released by injured tissue, and to HSP70. The corresponding box displays the emerging hypothesis that an upregulation of α7 nicotinic acetylcholine receptors (α7AChR) might also mediate immunosuppressive effects (see sect. III C).
mice lacking the neuronal/β-cell-type (Kir6.2/SUR1) K_{ATP} channels suggested that these channels are not obligatory for brain preconditioning (260). Further studies with improved pharmacological tools and transgenic mice are required to resolve this issue.

The recent demonstration that, in the hippocampus of gerbils, ischemic preconditioning decreased the binding density of cannabinoid CB1 receptors at 1, 2, and 4 days postpreconditioning deserves a mention (360), because CB1 receptor antagonists reduced the size of infarct and improved neurological function in the MCAO rat model (263). However, whether cannabinoids are neuroprotective or worsen neuronal damage after ischemia is still debated. For example, the CB1 agonist WIN 55212-2 was cerebroprotective in brain ischemia models (266), and the endogenous cannabinoid 2-arachidonoyl glycerol (2-AG) reduced brain damage in mice subjected to closed head injury (301).

To conclude this section, the ischemia-tolerant brain appears to be characterized by a shift of the neuronal excitatory/inhibitory balance toward inhibition. This potentially neuroprotective change does not appear to be effective during the test insult, because delayed latency of anoxic/ischemic depolarization is neither a common nor an effective neuroprotective change does not appear to be effective during the test insult, because delayed latency of anoxic/ischemic depolarization is neither a common nor a remarkable feature of the ischemia-tolerant brain (see sect. IIA); this would be expected if, during a severe ischemic insult, synaptic transmission is already "shut-down" early and efficiently in the naive brain. Rather, enhanced neuronal inhibition may protect the tolerant brain against posts ischemic hyperexcitability.

IV. FEATURES OF BRAIN TOLERANCE AT TISSUE LEVEL

A. Inflammatory Mechanisms

Focal cerebral ischemia elicits a strong inflammatory response through the activation of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 (35), involving both the brain's endogenous inflammatory cells (i.e., microglia and astrocytes) and peripheral leukocytes (151, 217, 358, 459). It is generally considered that posts ischemic inflammation contributes to infarct progression and worsens neurologic deficit, but it remains unclear whether the detrimental effects of inflammation (i.e., altered microcirculation, increased vascular permeability, adherence and extravasation of leukocytes into brain parenchyma) actually outweigh its neuroprotective and beneficial processes such as rapid debris removal and tissue repair (for review, see Ref. 391). Indeed, studying the contribution of cytokines to neurodegeneration is very challenging because the mechanisms involved are complex, with cytokines acting at very low concentrations on numerous cell types within and outside the brain, and mediating interdependent pathways that may be pro- or anti-inflammatory (52, 465; for review, see Ref. 5). Nevertheless, blocking the leukocyte adhesion to endothelial cells and their infiltration in brain tissue reduced the volume of infarct after transient MCAO (167, 424, 445, 449; for earlier studies, see Ref. 391), and the studies outlined next suggested that preconditioning-induced delayed tolerance was associated with a reduced inflammatory response to the subsequent ischemia. LPS preconditioning reduced ischemic brain injury in a mouse model of stroke, and this protective effect was associated with suppressed neutrophil infiltration and microglial/macrophage activation in the ischemic hemisphere, and reduced monocyte activation in peripheral blood (342). Preconditioning, induced 3 days before 90-min MCAO by either brief MCAO or injection of the mitochondrial toxin 3-nitropipionic acid, reduced the infarct volume as well as the posts ischemic expression of IL-1β and IL-6 (307). Ischemic preconditioning 3 days before 1-h MCAO in rats reduced the postischemic increased expression of several inflammatory genes, prevented the infiltration of neutrophils and macrophages in the ischemic lesion, and decreased the infarct volume produced by a subsequent 1-h MCAO (43). These studies clearly demonstrated that the reduction of ischemic lesion was associated with suppressed inflammatory response, but whether the latter contributed effectively to, or was a consequence of, reduced ischemic damage was difficult to ascertain in vivo. Within this context, the study of Zahler et al. (452) is especially pertinent; when cultured endothelial cells were preconditioned by a transient oxidative stress, their inflammatory responses to TNF-α [i.e., expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and E-selectin, and release of the pro-inflammatory cytokines IL-6 and IL-8] were markedly suppressed. Such actions of preconditioning at the endothelial interface may account for the antiadhesive/extravasation effects of preconditioning, and its protection of the microvasculature and BBB (see sect. ivC).

The potential mechanisms leading to, and underlying, the reduced inflammatory responsiveness that is associated with ischemic tolerance were reviewed by Karikó et al. (188). Through their analysis, these investigators argued that preconditioning produces a mild inflammatory response subsequent to Toll-like receptor (TLR) activation, which ultimately results in the production of several negative-feedback inhibitors of inflammation (Fig. 3). For the purpose of this review, this theory is outlined and discussed herein in simplified terms, with emphasis placed on selected and recent data.

Paradoxically, at first brain preconditioning elicits inflammation through activation of the proinflammatory transcription factor nuclear factor κB (NFκB) and induction of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (188), i.e., apparently through the same
mechanisms as those initiated by a severe ischemic insult, albeit with a response of lesser intensity. This initial, proinflammatory step is required for the development of ischemic tolerance: 1) inhibition of NFκB, by pretreatment with either the NFκB inhibitor diethylthiocarbamate or a NFκB oligonucleotide decoy, abolished the neuroprotective effects of different preconditioning methods against ischemia and kainate neurotoxicity (38); 2) treatment with a recombinant human IL-1 receptor antagonist (IL-1ra) blocked the development of ischemic tolerance induced in gerbils by 2 min of global ischemia (289); and 3) BB-1101, an inhibitor of MMPs, and TNF-α convertase (TACE) blocked the release of TNF-α caused by ischemic preconditioning and inhibited the development of ischemic tolerance (56).

The initial trigger of the inflammatory response induced by preconditioning is an interesting issue. TLRs are transmembrane receptors that recognize pathogen-associated molecular patterns (PAMPs) (80), thereby acting as pathogen sensors. However, some TLRs respond also to molecules released by injured tissue (30, 304), and a variety of TLRs are expressed in the brain, especially in microglia and astrocytes (177, 197). With a severe ischemic insult, it is clear that a variety of molecules derived from dead cells or damaged blood vessels may act as endogenous TLR ligands and initiate a strong inflammatory response. TLR activation is also to be expected with LPS-induced preconditioning, as LPS is a TLR ligand (251). However, the origin of TLR activation is less obvious with preconditioning stimuli that are reputed not to induce any cellular injury (e.g., cortical spreading depression). In the latter cases, TLR activation may be due to HSP70, since it was identified as an endogenous stimulus for inflammatory responses mediated by TLR2 and TLR4 (16, 336, 421), and upregulated HSP expression is a consistent feature of preconditioning (see sect. uD). As TLRs are sensors for extracellular ligands, this would imply a potential role for HSP70 at extracellular level, although some data suggest that there may not be a direct ligand/receptor interaction between HSP70 and TLR2/4 (410).

With regard to the subsequent, reduced inflammatory responsiveness that contributes to ischemic tolerance, several negative regulators of inflammation appear to be involved (Fig. 3; for review, see Ref. 188). By acting at the extracellular level, anti-inflammatory cytokines (e.g., IL-10), cytokine receptor antagonists (e.g., IL-1ra), and decoy receptors (e.g., IL-1RII) may limit the progression of inflammation away from the damaged brain regions, whereas intracellular inhibitors (e.g., TTP and SOCS-3) may reduce the intensity of the inflammatory response. Although this concept of multifactorial, negative regulation of inflammation with ischemic tolerance is novel, it is already supported by convincing data.

IL-10 has been identified as an anti-inflammatory cytokine that can limit inflammation through various actions: 1) inhibition of the synthesis of a variety of proinflammatory cytokines, including TNF-α, IL-1β, and IL-8; 2) suppression of cytokine receptor expression; 3) inhibition of cytokine receptor activation; and 4) mediator of cell-mediated immunomodulation. IL-10 is synthesized in the CNS, and IL-10 receptors have been demonstrated on microglia, astrocytes, and oligodendrocytes (392). Although there may be no report yet on an effective role of IL-10 in brain ischemic tolerance, several lines of evidence support this possibility. Endotoxin preconditioning enhanced IL-10 renal expression in rats, and this change correlated with ischemic tolerance (140). In the liver of wild-type mice, hypertonic preconditioning increased IL-10 expression and prevented ischemia/reperfusion injury; ischemic tolerance was suppressed in IL-10 knockout mice (296). In cultured microglia and astrocytes, the expression of IL-10 mRNA as well as the production of IL-10 protein was enhanced by innate activation with LPS (52, 254). Finally, procedures that increased IL-10 were cerebroprotective in two separate studies: 1) intracerebroventricular or systemic administration of IL-10 decreased the infarct size produced in rats by MCAO (381), and 2) when viral vectors encoding human IL-10 were injected in the lateral ventricle of rats, 60 or 90 min after focal ischemia, the infarct volume was smaller and the inflammatory response reduced. The same procedure also protected hippocampal neurons against global ischemia, a beneficial effect that was associated with attenuated IL-1β, but increased TNF-α (294).

IL-1ra is an endogenous protein acting as a highly selective, competitive antagonist of the IL-1 receptor type I (IL-1R1), the receptor through which IL-1β exerts its proinflammatory actions (13, 175). IL-1ra mRNA is constitutively expressed in the brain, and three IL-1ra protein isoforms (secreted IL-1ra and intracellular IL-1ra type 1 and 2) were upregulated in response to LPS treatment (300). Ischemic tolerance induced by 10 min of MCAO in rats was associated with increased IL-1ra mRNA and protein expression (23), and several studies suggest that increased IL-1ra contributed effectively to ischemic tolerance. Intracerebroventricular or peripheral injection of IL-1ra reduced ischemic brain damage in rodents (130, 231, 393), and IL-1ra was still effective when it was administered as late as 3 h after 60-min MCAO (259). Furthermore, endogenous IL-1ra was demonstrated to be neuroprotective by itself, as IL-1ra knockout mice exhibited infarcts produced by transient MCAO that were 3.6-fold larger than in wild-type animals (315). Reciprocally, IL-1ra overexpression, whether induced by adenoviral gene transfer or drug associated, protected rats against cerebral ischemia (36, 302) and attenuated the ischemic inflammatory response in the mouse brain (445).

True decoy receptors are defined as cell surface ligand-binding proteins with high affinity and specificity, but which neither induce downstream signaling nor
present ligand to a signaling receptor. Soluble “decoy receptors” (e.g., released by cell-surface shedding) also exist (236, 237). The IL-1 type II receptor (transmembrane IL-1RII or soluble sIL-1RII) was the first pure decoy to be identified, with others subsequently discovered for members of the TNF-R and IL-1R families. IL1-RII mRNA expression was detected in cultured astrocytes and increased by LPS treatment (314), but there is apparently no report directly related to the potential role of IL1-RII in brain ischemic tolerance. Nevertheless, some data support this possibility. When IL-1β was microinjected into the brain of rats to generate an inflammatory response without neuronal death, this procedure induced an increase of de novo IL-1β synthesis with a concomitant increase of IL-1RI (i.e., the IL-1/β signaling receptor), but there was also a much greater increase of IL-1RII (i.e., IL-1β decoy receptor) with immunostaining, indicating that IL-1RII was expressed on brain endothelial cells and on infiltrating neutrophils (95). In two studies with rodents subjected to MCAO, the low basal level of IL-1RII mRNA in the cortex was markedly elevated after ischemia (413, 430).

Among the numerous intracellular and transmembrane inhibitor proteins involved in negative regulation of TLR-mediated immune responses (188, 227), only the potential role of SOCS-3 (suppressor of cytokine signaling-3, a negative regulator of cytokine signaling, especially of IL-6; Ref. 4) and tristetraprolin (TTP, a component of a negative-feedback loop that interferes with TNF-α production by destabilizing its mRNA; Ref. 55) were investigated so far. Application of hypertonic salt solutions to the cerebral cortex of rats, a procedure that increases ischemic tolerance, produced a sustained increase in the levels of SOCS-3 and TTP mRNA (261). Previous data also support the notion that these cytosolic inhibitors of inflammation may contribute to ischemic tolerance. Transient MCAO produced an upregulation of the SOCS-3 gene in the rat cerebral cortex, and antisense knockdown of ischemia-induced SOCS-3 increased the infarct volume, suggesting a neuroprotective role for SOCS-3 (329). A marked and sustained upregulation of SOCS-3 was also observed in cortices of rats subjected to permanent MCAO (25). With regard to TTP, the expression of its gene was upregulated in the canine myocardium 16 h after ischemic preconditioning (467).

To conclude this section, preconditioning appears to induce, secondarily, the expression of several endogenous inhibitors of inflammation that reduced the brain inflammatory responsiveness, a condition that may be an important feature of ischemic tolerance. Interestingly, a similar state can be produced by mucosal vaccination with pertinent antigens. Mucosal (nasal or oral) antigen administration, when appropriately dosed and scheduled, preferentially induces regulatory T cells that secrete the anti-inflammatory IL-10 and TGF-β at the anatomic site where the administered antigen is located. In simpler terms, this process (termed bystander suppression) leads to a relatively organ-specific immunosuppression. In several studies, CNS antigens were used to test whether this type of immunologic tolerance (i.e., induction of antigen-specific mucosal tolerance for suppression of cell-mediated autoimmune responses) could protect against a subsequent ischemic insult. Rats fed five times over 2 wk with 1 mg bovine myelin basic protein (MBP) had smaller infarcts after transient MCAO (induced 2 days following the last antigen feeding); this immunologic tolerance and its neuroprotective effects were associated with an increased expression of TGF-β, and were transferred to naive animals that received splenocytes from MBP-tolerized donors (27, 28). In mice, nasal tolerance to myelin oligodendrocyte glycoprotein (MOG; applied 3 times every other day) decreased by 70% the size of infarct (MCAO induced 2 days after the last nasal administration) and reduced also the neurological deficit; this protective effect, which was associated with a marked increase in the anti-inflammatory cytokine IL-10 (but not of TGF-β), was absent in IL-10 knockout mice (116, 117). Similar results were obtained with nasal tolerance to E-selectin, a cytokine-inducible adhesion molecule restricted to blood vessel endothelium activated by inflammatory stimuli (68).

It is pertinent to note that whether these “tolerization strategies” act primarily at brain tissue level or on the adaptive immune system is an important, unanswered question. Clearly, the interactions between CNS and immune system are finely balanced (466), and if brain injuries are capable of inducing immunodepression (247), neuroprotection may be achievable via the adaptive immune system. Other peripheral systems (e.g., cardiovascular, autonomic nervous system) may also contribute to ischemic tolerance. One example is coagulation. 1) In mice, 3 days after preconditioning by 15 min of focal ischemia, the vasoconstrictor and platelet aggregation inhibitor cyclooxygenase-1 was upregulated, and bleeding times were about 5 times longer than in controls (387). 2) Hypocoagulation is a feature of hibernation, thought to reduce the risk of thrombus formation associated with the very low blood flow levels that are associated with this condition (97).
olism and brain cell membrane ionic homeostasis (see sect. \( \text{II} \)). There is convincing evidence that the cerebrovascular system has such an adaptive capability through arteriogenesis (formation of collaterals from existing arteries; Ref. 156) and angiogenesis (capillary proliferation; Ref. 145). When both carotid arteries are ligated in rats to produce a chronic hypoperfusion, cerebral blood flow (CBF) drops sharply right after the occlusion, but then increases gradually during the following weeks (110, 298), and recent studies confirmed that both arteriogenesis and angiogenesis may contribute to this adaptive process (72, 286).

2. Induction of VEGF by preconditioning

VEGF is a key regulator of angiogenesis (111, 389), another target of HIF-1 besides GLUT1 (see sect. \( \text{II} \)) (362), and brain VEGF was upregulated early after hypoxic preconditioning (exposure to 8% \( \text{O}_2 \)) in both neonatal and adult rats (34, 406). The induction of VEGF gene expression was also demonstrated after MCA occlusion in rats and mice, both in the infarct and peri-infarct regions (155, 318, 429), but contradictory data were also reported (222). These data suggest that angiogenesis may be initiated at an early stage after preconditioning, at least with some stimuli, but the ultimate contribution of VEGF upregulation to ischemic tolerance through angiogenesis is difficult to assess: 1) because it is well established that VEGF-induced angiogenesis is associated with a marked increase in vascular permeability (82, 102), which implies that VEGF upregulation may exacerbate ischemia-induced BBB disruption and edema (457); and 2) because VEGF is also an important signaling molecule in the CNS capable of suppressing apoptosis and stimulating neurogenesis (145).

3. Residual CBF during the test ischemia: is it improved by ischemic tolerance?

Several studies examined how chronic hypoperfusion or preconditioning altered residual CBF during a subsequent test ischemia, but the results are conflicting.

In mice, Kitagawa et al. (204) showed that 14 days of chronic mild reduction of cerebral perfusion pressure, produced by unilateral carotid artery ligation, were necessary for efficient collateral development (i.e., preservation of cortical perfusion after MCA occlusion). Such a delay is well beyond the time window of ischemic tolerance induced by brain preconditioning (generally 1–7 days). However, in an earlier study carried out with rats and using forebrain ischemia as subsequent test insult, both residual CBF and ATP levels in the parietal cortex were improved significantly at 3 and 7 days after unilateral carotid artery ligation (47).

In the study of Matsushima and Hakim (243) with rats, a 2- or 3-h test MCAO was preceded 4 days earlier by 30-min MCAO, 5-min global ischemia, or sham surgery. CBF was improved significantly in the frontoparietal cortex during the test MCAO when rats were subjected to the prior focal ischemia, but this change was not associated with a reduction in infarct volume. In contrast, the infarct volume was significantly reduced when MCAO was preceded by global ischemia, but without any change in residual CBF. Similar, negative data (i.e., effective ischemic tolerance without change in CBF during the subsequent test MCAO) were reported by Chen et al. (66).

In contrast to the studies outlined in section \( \text{II} \), data obtained with CSD preconditioning in rats and ischemic preconditioning in gerbils suggested that ischemic tolerance was associated with improved tissue perfusion during the subsequent test ischemia (269, 297).

With LPS preconditioning in rats, Dawson et al. (89) reported that local CBF was not significantly different (relative to saline control) at 15 min after permanent MCAO, but that microvascular perfusion was later improved in LPS-treated rats, i.e., at 4 and 24 h after MCAO. More recently, the same procedure was found to improve CBF in the peri-infarct area during the test MCAO (127).

Overall, the relatively long delay necessary for effective cerebrovascular remodeling appears difficult to reconcile with the window of ischemic tolerance (i.e., 1–7 days post-preconditioning), and residual CBF does not appear to be consistently improved in tolerant regions early during the test ischemia.

4. Does preservation of the vascular reactivity after the test ischemia contribute to ischemic tolerance?

More important for ischemic tolerance may be the preservation of vascular control/reactivity as the physiologic response of ischemic damage progresses, especially the endothelium-dependent arterial relaxation to acetylcholine that is selectively impaired by ischemia-reperfusion (76, 341) and hypoxia-reoxygenation in vitro (383). In support of this notion, cerebral endothelial cells could be preconditioned in vitro (8), HSP70 expression in endothelial cells was associated with hypoxic-ischemic tolerance induced by hyperthermic preconditioning in immature rats (172), and the vascular endothelium was protected in ischemia-tolerant regions (see also sect. \( \text{V} \)). Ischemic preconditioning in rats improved CBF during the reperfusion that followed the subsequent 30-min forebrain ischemia, and this functional effect was associated with reduced endothelial desquamation and brain edema (426). An upregulation of endothelial NOS (eNOS) may also contribute to improved recirculation in tolerant brain regions, since increased expression of eNOS mRNA was associated with ischemic tolerance induced by ischemic or LPS preconditioning (127, 152, 127). The protective effect of preconditioning (at least when it is induced by LPS) on cerebrovascular reactivity may also include an
action on vascular smooth muscles, because this treatment prior to ischemia-reperfusion prevented both the impairment of endothelium-dependent relaxation to acetylcholine and the reduction in potassium inward rectifier (Kir) density measured in dissociated smooth muscle cells dissected from the occluded MCA (24).

C. BBB Integrity

The BBB is anatomically defined by the tight junctions between adjacent endothelial cells lining the lumen of cerebral microvessels, but the BBB function also involves astrocytes, pericytes, neurons, and the extracellular matrix. The latter constitutes the basement membrane underlying the vasculature, and its disruption is strongly associated with increased BBB permeability in pathological states (154). BBB breakdown with associated vasogenic edema is an important and early feature of ischemic brain damage and appears as a precursor and a good predictor of poor outcome (216). Several studies have shown that ischemic tolerance includes a better preservation of the BBB and reduced edema formation during the test ischemia. With preconditioning induced by 15-min MCAO and 3 days before the test ischemia in rats, both BBB disruption and edema measured 24 h after permanent MCAO were attenuated in perilesion regions, but not in the ischemic core (240). The latter data were confirmed by the more recent study of Zhang et al. (454). When immature rats were subjected to 2 h of hypoxia-ischemia, IgG extravasation indicating BBB disruption was closely associated with early neuronal damage, and both were significantly attenuated by hyperthermic preconditioning (173). Here again, it is difficult to determine whether the preservation of BBB integrity contributes to, or is a consequence of, reduced ischemic damage. From mechanistic point of view, it is clear that a reduction of the brain inflammatory responsiveness (see sect. IV) is likely to help maintain BBB integrity. However, preservation of BBB integrity may be a primary and important feature of ischemic tolerance, given that it is the interface between damaged/vulnerable tissue and circulating inflammatory cells during recirculation. This notion is supported by at least two separate lines of evidence.

MMPs, especially MMP-9, are known to degrade the neurovascular matrix during reperfusion, thereby contributing to BBB breakdown (339, 446, 458). Both BBB disruption and MMP-9 expression were reduced when MCAO was preceded by ischemic preconditioning in rats (454), a protective effect to which HSP70 may contribute as HSP overexpression suppresses MMP-9. As already mentioned, inhibition of MMP-9 has protective effects in stroke models (see sect. IV for further information and references on HSP70 and MMPs).

The adhesion molecules expressed by endothelial cells (e.g., ICAM-1) mediate the firm adhesion of leukocytes to vascular bed, but also trigger signaling cascades that promote BBB leakage and leukocyte infiltration and MMP-9 expression were reduced when MCAO was preceded by ischemic preconditioning in rats (454), a protective effect to which HSP70 may contribute as HSP overexpression suppresses MMP-9. As already mentioned, inhibition of MMP-9 has protective effects in stroke models (see sect. IV for further information and references on HSP70 and MMPs).

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V. CONCLUDING REMARKS

As preconditioning implies pretreatment, and the severity of an ischemic insult depends on the balance between energy demand and supply, ischemic tolerance could include adaptive mechanisms to reduce energy demand (e.g., through changes in voltage- and/or ligand-operated ion channels) or improve residual energy supply (e.g., collateral blood supply). However, on the basis of the information assembled in sections IV (energy metabolism and cellular ionic homeostasis) and V (cerebrovascular adaptation), this type of adaptation may not contribute significantly to preconditioning-induced ischemic tolerance. When a cerebral artery is occluded in the tolerant brain, the severity of the resultant insult appears to be the same as in the naive brain, but the resulting neuronal damage is substantially less. Overall, the data available so far suggest that the adaptive changes in the tolerant brain may be directed primarily against posts ischemic and delayed deleterious processes that contribute to ischemic damage. However, adaptive changes that are beneficial during the subsequent lethal ischemia cannot be ruled out, and the possibility that the tolerant brain may have an improved ability to control intracellular Ca2+ overload deserves further studies (see data on cytosolic Ca2+ homeostasis in sect. IV).

Interrelated, multifactorial processes are responsible for ischemic tolerance (Fig. 4). This is a key feature of adaptive cytoprotection that appears throughout this analysis. Does it imply that several cytoprotective processes must be activated for effective protection against ischemic damage, or is there some degree of redundancy among these endogenous processes? On the one hand, in several studies, the reproduction of only one feature identified in the tolerant brain (e.g., through genetic manipulation) induced a significant cerebroprotection. On the other hand, the former notion agrees with the fact that ischemic brain damage involves multiple, parallel, and sequential pathophysiological events. Indeed, it is now recognized that the suppression of a single deleterious cascade may not be sufficient for effective protection...
against ischemic damage, and a number of investigators are now working on combination therapies (213, 359, 378).

Combination therapy is inherently difficult for drug development because of possible drug-drug interaction and adverse effect superimposition, and this is where an improved knowledge of the molecular physiology of ischemic tolerance is likely to help. Several innovative neuroprotective strategies with combination therapeutic potential are already emerging from this field of study.

An obvious approach is to activate one of the sensors that is initially activated by preconditioning, such as HIF-1 (323, 371), as this will induce the transcription of several target genes (GLU-1, VEGF, and EPO in the case of HIF-1) and ultimately contribute to several neuroprotective components of ischemic tolerance. However, this strategy may have a potential drawback: overstimulation of the targeted preconditioning sensor may mimic the effect of a damaging stressor, thereby initiating cell death rather than protecting it.
than tolerance. Indeed, as pointed out by Dirnagl et al.
(94), preconditioning/ischemic tolerance is part of a con-
tinuum spectrum of responses to noxious stimuli, and
although the tissue response (i.e., tolerance or cell death)
depends on the stimulus intensity, there are no clear
boundaries between tolerance, apoptosis, and necrosis.
In other words, pharmacological activation of precondition-
ing sensors may lead to tolerance only when drugs are
administered within a narrow range of concentrations.

An alternative is to interfere with a transducer (i.e.,
downstream from the preconditioning sensors), and this
is the case of EPO (93), which is attracting much interest
because this adaptive response is robust and consistently
achieved at effector level, i.e., with molecular targets
already approved for the treatment of various conditions
as a potential neuroprotector (94, 104), partly because it is
already approved for the treatment of various conditions
in humans.

Combinatorial neuroprotective strategies may be
also achieved at effector level, i.e., with molecular targets
underlying ischemic tolerance. The most obvious target of
this type is HSP70 because its upregulation results in
multiple, beneficial actions (Fig. 1) (380, 460). Although
mKATP channels are not involved in multiple, endogenous
neuroprotective processes (Fig. 4), it is also a pertinent
target because its opening results in the preservation of
mitochondria membrane potential and function, which
then leads to multiple benefits (e.g., attenuation of mito-
chondrial Ca\(^{2+}\) overload, reduced mitochondrial ROS
generation, reduced release of proapoptotic factors). It is
important and timely to investigate further the role of
mKATP channels in brain ischemic tolerance. Finally, the
thioredoxin (Trx) system deserves a mention because, in
addition to its contribution to cellular defense against
ROS, it is emerging as a key regulator of fundamental
cellular processes (see sect. ivC), and the antidepressant
deprenyl upregulated Trx in vitro (11).

Although mimicking the reduced inflammatory re-
sponsiveness of the tolerant brain cannot be assimilated
to a combination therapy, this strategy is worth pursuing
because this adaptive response is robust and consistently
induced by a variety of preconditioning stimuli. Among
others, and rightly so, the mucosal vaccination strategy
for neuroprotection (see sect. ivA) is triggering much
interest because of its potential, direct applicability to
clinical practice.

With regard to clinical relevance, the earliest clinical
applications of our improved knowledge of ischemic tol-
erance are likely to be prophylactic treatments to mini-
mize the risk of neurological deficits that are associated
with ischemic insults, especially those that can be antici-
ipated (i.e., essentially surgery related). For example, cor-
onary artery bypass surgery has become a common, life-
saving procedure, but potential adverse effects do include
neurological damage and cognitive impairment (310). Patients with a history of TIA constitute another large
group who may benefit from prophylactic neuroprotec-

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Address for reprint requests and other correspondence:
T. P. Obrenovitch, Prof. of Neuroscience, Div. of Pharmacology,
School of Life Sciences, Univ. of Bradford, Bradford BD7 1DP,
UK (e-mail: t.Obrenovitch@bradford.ac.uk).

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