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14-3-3γ and Neuroglobin are New Intrinsic Protective Factors for Cerebral Ischemia

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Abstract A number of intrinsic factors are present intracellularly and could be turned on to protect cells from stress and injury, including cerebral ischemia. The degree of protection of these factors is dependent on the time of induction, their concentration, as well as the duration and extent of injury. This review summarizes recent studies on some of the protective factors with specific emphasis on two recently discovered intrinsic protective proteins: 14-3-3γ protein and neuroglobin. Both of them were originally discovered in neurons, later identified in astrocytes under ischemic conditions, and demonstrated to have protective effect on nerve cells from apoptosis. Understanding the mode of induction and role of protection of these intrinsic protective proteins would be beneficial for the future development of pharmacotherapy in extending the therapeutic time window, which would lead to better stroke management for patients.

Keywords Intrinsic protective protein · Stroke · Preconditioning · 14-3-3γ · Neuroglobin

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Introduction

Cerebral ischemic stroke remains the second most common cause of death and the major cause of disability in the world. The threat of stroke continues to greatly affect people's quality of life, and so far, an effective therapy has yet to be found. The effectiveness of existing therapies was limited by the shortness of therapeutic time window [1]; that is, the time frame within which successful therapy can be initiated prior to onset of irreversible injury [2]. Therapies initiated within this time frame have a greater chance of abolishing the cause of injury and reducing irreversible injury. Hence, studies in determining the length of the therapeutic time window have become essential to the development of effective stroke therapy. A number of studies utilizing animal models support the existence of the therapeutic time window of approximately 2–4 h after the onset of stroke [3, 4]. For human stroke patients, accurate estimations of the therapeutic time window have not been confirmed yet. However, the recommended practice for all clinical interventions after the onset of stroke is within 6–8 h of the injury. The therapeutic outcome becomes weakened considerably after 12–24 h, and damage is

practically irreversible after 48 h [1, 5]. Great difficulties exist for stroke patients to receive timely clinical attention and for doctors to deliver suitable treatments within 3–6 h of a stroke attack. Therefore, researches into finding new means to extend the therapeutic time window are of urgently needed.

There is accumulated evidences demonstrating that many endogenous protective mechanisms are activated during early phase of ischemic injury. Up-regulating the expression of protective factors has the potential to extend the effective therapeutic time window. For example, ischemic preconditioning has been proven to have protective effects against subsequent severe ischemic injury through modulating endogenous molecules in in vivo animal models such as dog [6], mouse [7], and gerbil [8], and also in in vitro models [9]. Further investigations also show that ischemic preconditioning is associated with the up-regulation of many innate protective factors, thus elevating the tolerance of cells and organisms to stroke damage. This review examines the current researches of intrinsic protective proteins known to be related to ischemia. We also focus our discussion on the recent discovery of two intrinsic protective proteins, 14-3-3γ and neuroglobin (Ngb).

Stroke Treatments

Stroke is a serious threat to human health. Even though medical science has greatly advanced over the past few decades, the development of an effective stroke treatment has remained elusive [10]. Hankey et al. [11] studied stroke patients in Western Australia and reported that the risk of death is about 25% within a month, 33% after 1 year, and a further 15% over the next 5 years. In China, it was estimated that 1.5–2 million patients suffer from stroke every year [12].

Since 1995, intravenous thrombolytic treatment with recombinant tissue plasminogen activator (rt-PA) has been a recommended medical therapy for acute ischemic stroke. To obtain its optimal therapeutic effect, one should apply treatment within 6 h after stroke onset [13]. Subsequent research emphasized that thrombolytic treatment within 3 h after stroke onset increased the chances of receiving minimal or no disability after 3 months by at least 30% [14]. If thrombolysis is implemented within 90 min, the outcome is further improved [15]. However, thrombolytic agents increase bleeding and require careful monitoring, and the risk of reperfusion injury can rise drastically if used beyond 3 hours of stroke onset.

Thrombolytic therapy has been a widely accepted treatment for acute stroke treatment. Pharmacotherapy for treating high-risk stroke patients also include antiplatelet drugs (such as aspirin, clopidogrel, dipyridamol, and ticlopidine), anticoagulants (heparin and warfarin), and neuroprotective agents (like calcium antagonist and antioxidant). However, the risk of bleeding increases with the increasing time of ischemia. Moreover, the resulting benefit is minimal, and approximately 9 patients per 1,000 treated are saved from death or disability, while prevention occurs in only 4 per 1,000 [16–18].

The limitations in therapeutic benefit and unsatisfactory prognosis lead us to explore methods for extending therapeutic time window. Extension of therapeutic time window would provide a better opportunity for high-risk groups of stroke patients to receive successful treatment. Wahlgren et al. [19] reported that the therapeutic time window could be extended to 4.5 h for effective thrombolytic treatment. The European Cooperative Acute Stroke Study III (ECASS III) also agrees that earlier treatment increases the chances of recovery, reaffirming the need for rapid delivery of therapy after stroke onset [20]. If there are factors capable of slowing down the pathoprogression of stroke and lengthening the therapeutic window, these treatments would become more beneficial.

Preconditioning Triggers Intrinsic Protection

"Natural forces within us are the true healer of disease!" Hippocrates said. Creatures of all sizes including their cells have innate defensive system of intrinsic protective factors to guard themselves against unexpected stresses and injuries. At the cellular level, cell death occurs when these intrinsic protective factors are overwhelmed to a stage beyond their ability to protect and repair. Preconditioning is an endogenous strategy that leads cells and organisms to initiate the expression of these intrinsic factors to acquire tolerance, so as to defend themselves from subsequent damage. The term of preconditioning was introduced by Janoff in 1964 [21]. Murry et al. [6] reported ischemic preconditioning in myocardium using an in vivo dog model and found multiple brief ischemic episodes protecting cells from a subsequent and sustained insult. Similar phenomenon was observed in gerbil's brain tissue. A single 2-min ischemia given 1 or 2 days before 5-min ischemia exhibited partial protective effects. On the other hand, two 2-min ischemic treatment intervals in 2 days showed complete protection against neuronal death [8]. Although the exact mechanisms are not clear yet, preconditioning is considered a powerful adaptive intrinsic defense reflecting the existence of an endogenous program of neuroprotection. These confirmed preconditioning occurred in response to sublethal ischemia, stresses, and injuries. Its protective functions might involve inhibition of proapoptosis pathway and promotion of prosurvival pathway. This would be achieved by the induction and expression of intrinsic protective factors such as proteins, lipids, hormones, etc. [22–24].

Preconditioning may consist of early and late phases. It depends on whether the effect appears immediately after the sublethal injury or not. Early effects are considered to be related to the adaptation of membrane receptors and activation of ion channels. Late effects are the results of regulation of gene and protein expression [25]. Cerebral ischemia is known to cause an early cell depolarization, leading to neurotransmitter release and cytotoxicity as well as a number of subsequent pathophysiological changes. Many immediate early genes and transcription factors appear to be transiently and rapidly activated in response to ischemia. The activation of immediate early genes and transcription factors in early phase of injury is not mainly mediating cell death, but most of them may serve in reducing cell damage and promoting cell survival, thus acquired neuroprotection [26]. Recent findings confirmed that preconditioning can alter the expression of immediate early genes under ischemia injury, thus enhancing the protective and adaptive responses to ischemia at the gene level [27]. Genomic studies in rat [28] and mouse [29] have further demonstrated that neuroprotection induced by preconditioning involves reprogramming of gene expression. Ischemic preconditioning was found to inhibit p53 and Bax expression in rat neuron, thus contributing to the protection observed in the CA1 region of hippocampus against apoptosis caused by ischemia/reperfusion [30]. Many proteins are also found to be induced by preconditioning to prevent cell dysfunction. For example, hypoxia-inducible factor-1 (HIF-1) and erythropoietin (EPO) expression increased greatly after hypoxia and reached even higher levels with hypoxia preconditioning, exerting neuroprotection through the Jak2/Stat5 and nuclear factor-κB (NF-κB) pathways [31].

At present, there is hardly any neuropathology data on changes of human brains before and after preconditioning or preconditioning-like ischemic periods. Transient ischemic attacks (TIAs) were considered clinically correlated to preconditioning. It has been defined as "a brief episode of neurologic dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting less than 1 hr, and without evidence of acute infarction" [32]. A study on prior cerebral ischemic episode (stroke or TIA) reported that TIA but not stroke in the patient history was associated with decreased odds for in-hospital case fatality [33]. Since the phenomenon that ischemic preconditioning as induction of ischemic tolerance after TIA in human brain was proven to be clinical relevant, the beneficial effect of TIA suggests that inducible endogenous neuroprotection also exists in the human brain [34, 35]. Remote ischemic preconditioning refers to the concept that transient ischemia, happened in a site distance from heart or brain such as a limb, can lead to protection of the myocardium and possibly the brain. Remote ischemic preconditioning was used as a way to mimic preconditioning and found to increase myocardial salvage with a favorable safety profile from the beginning of acute myocardial infarction up to hospital admission [36]. Additionally, increasing number of clinical trials has been carried out to test the safety and effectiveness of preconditioning-derived strategies on protecting brain from irreversible damage. These include remote ischemic preconditioning decreased subclinical cerebral and myocardial damage in carotid endarterectomy patients [37] and pharmacologic preconditioning in preventing ischemic neurologic deficits and consequences [38]. Preconditioning is accepted as an invaluable and powerful technique based on neuroprotection research and provides new insight of clinical managements, although there are still many uncertainties to be clarified.

The intrinsic protection induced under preconditioning led to a promise for the development of an alternative strategy of protection based on the exploitation of the brain's own intrinsic protective mechanisms. A schematic diagram that illustrated the principle components of therapeutic time window is shown in Fig. 1. Clarification of the mechanisms involved in protective factor induction by preconditioning would lead to probable future strategies on how to manipulate these intrinsic factors in extending the therapeutic time window. This new concept relies on the evidence that the brain tissue is able to protect itself by way of short-term or long-term adaptation to transient episodes of ischemia preceding sustained ischemia. A number of protective mechanisms are activated immediately after mild ischemic injury or preconditioning. Subsequently, the level of these protective factors increases to a level higher than the threshold, when they begin to exhibit neuroprotective function. The period with the level of protective factors in the brain being maintained above the threshold is the therapeutic time window. Pharmacotherapy applied in this time frame will eliminate the cause of damage and prevent further injury being developed, thus allowing tissue repair. If therapy was not applied on time, ischemic injury will continue to inflict damage and the effect of protective factors would be overwhelmed; thus, the inflicted damage becomes irreversible. In other words, if a brief ischemia raises the intrinsic protective factors above the tolerance but without causing any actual damage, the tissue will be protected from the next attack if these factors remain above the tolerant level. Understanding the mechanism of induction of these protective factors at early stage of insult would guide us to the development of pharmacologic modulations of these innate protective factors that might mimic protective effects of preconditioning and thus provide a safer way of inducing cerebral ischemic protection in humans. This might also help in lengthening the therapeutic time window, elevating the resistance to stroke, and decreasing the mortality in high-risk stroke patients.

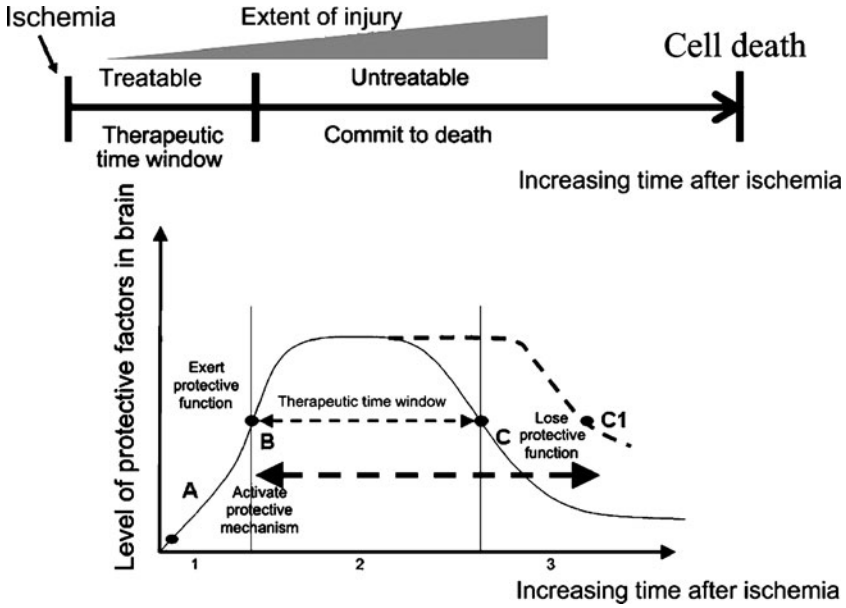

Fig. 1 Representation of the time course of pathogenesis after stroke (upper panel). The course is separated into three phases, 1, 2, and 3. Phase 1: during this period, a number of protective mechanisms are activated immediately after mild ischemic episode or preconditioning (point A). Subsequently, when the level of protective factors increases to point B, i.e., a significant threshold concentration is accumulated and neuroprotection begins. Phase 2: during this period (between points B and C), the level of protective factors are maintained above the threshold, i.e., the therapeutic time window. Therapies initiated within this time frame will be beneficial to the patients. Phase 3: during this period, the ischemic injury continues to inflict damage on the cells and the protective capacity of these factors are overwhelmed or their levels begin to decrease. At point C, their levels have decreased below the threshold for effective protection and inflicted damage becomes irreversible. New methods, e.g., by enhancement of the expression of some intrinsic factors, could lengthen the therapeutic time window from point C to C1, thus allowing patients to receive proper treatment

Intrinsic Protective Factors

Intrinsic protective factors include various kinds of molecules such as lipids, hormones, transcriptional factors, endogenous protective proteins, etc. (Table 1). Lipid messengers and hormones were attracting more attentions after they were found having protective effect against oxidation. Cerebral ischemia leads to the cleavage of specific phospholipids by phospholipases to release lipid messengers from membrane. Oxidative stress disarranged lipid signaling will lead to neurodegeneration. Metabolism of polyunsaturated phospholipids in excitable membranes was found to be significantly sensitive to cerebral ischemia [39]. These lipid messengers and downstream pathways may play important roles in modulating signaling cascades, promoting, regeneration, repairing neurons, and regulating the integrity of neuronal, glial, and endothelial cell functions. Phospholipase A_2 (PLA_2) could be activated by hypoxia/ischemia, and this would lead to the accumulation of free arachidonic acid, docosahexenoic acids, and lysophospholipids. These bioactive lipids will in turn induce several downstream signaling pathways to synthesize platelet-activating factor (PAF) and ecosanoids (prostaglandins and leukotrienes) [22]. *N*-oleoyldopamine (OLDA) is a novel endogenous capsaicin-like lipid shown to protect the heart against ischemia–reperfusion injury due to activation of transient receptor potential V1 (TRPV1) [40]. However, whether OLDA can exert a similar protective role in the brain remains to be investigated.

The protective roles of hormones under ischemia also attract increasing attention. Insulin was found to contribute to the neuroprotective effect of remote mesenteric ischemic preconditioning [41]. Insulin-like growth factor-1 (IGF-1) was reported to exert neuroprotection against ischemia-induced neuronal death [42]. Endogenous estrogens and their receptors have been shown to play important roles beyond the reproductive system. A recent study indicated that endogenous estrogen 17β-estradiol could provide neuroprotective effects by enhancing neurotrophic support, suppressing proinflammatory pathways, and ultimately decreasing cell death [23]. Tixier et al. [43] reported that adrenomedullin could serve as a potent autocrine and paracrine neuroprotective factor and was involved in neuroprotection induced by endothelial cells and microglia during cerebral ischemia. Other hormones, such as progesterone [44] and melatonin [45], two natural derivatives of thyroxine, 3-iodothyronamine, and thyronamine [46], exerted protective effects under ischemia. However, the detailed mechanisms are not clearly defined yet.

Table 1 Intrinsic protective factors

Categorization	Intrinsic protective factors	References
Cytokine (chemokine and growth factor)	G-CSF	[97]
	bFGF	[98]
	VEGF	[99]
	SDF-1	[100]
	NGF	[101]
	EPO	[102]
	BDNF	[103]
	PEDF	[104]
	CNTF	[105]
	NT-4	[105]
	TGF-β1	[106]
	GDNF	[107]
	CX3CR1	[108]
Transcriptional factor	HIF-1α	[109]
	CREB	[110]
	c-fos	[111]
	PPAR-α	[112]
	PPAR-γ	[113]
	P53	[114]
Lipid	PAF	[22]
	Ecosanoids	[22]
	OLDA	[40]
Hormone	IGF-1	[42]
	17β-Estradiol	[23]
	Adrenomedullin	[43]
	Progesterone	[44]
	Melatonin	[45]
	3-Iodothyronamine	[46]
	Thyronamine	[46]
Antioxidant	CuZn-SOD	[115]
	GSH-Px	[116]
Neurotransmitter (or related receptor/transporter)	GABA and GABA receptor	[117, 118]
	GHB	[119]
	5-HT and its receptor	[120]
	Glut-1	[60]
	GluR6	[63]
Endogenous cytokine antagonist	IL-1ra	[121, 122]
Endogenous protective protein	HPGDS	[50]
	Ngb	[123]
	14-3-3γ	[71]
	Endocannabinoids	[124]
	Adenosine	[125]
	Bcl-2	[126]
	Hsp 70, Hsp 27	[127]
	NPD1	[48]
	UCP2	[51]
	Neuregulin-1	[52]
	NF-κB	[53]
	Kynurenic acid	[128]

G-CSF granulocyte colony-stimulating factor, *bFGF* basic fibroblast growth factor, *VEGF* vascular endothelial growth factor, *SDF-1* stromal cell-derived factor-1, *NGF* nerve growth factor, *TNFR1* tumor necrosis factor receptor 1, *EPO* erythropoietin, *BDNF* brain-derived neurotrophic factor, *PEDF* pigment epithelium-derived factor, *CNTF*, ciliary neurotrophic factor, *TGF-β1* transforming growth factor β1, *GDNF* glial cell-derived neurotrophic factor, *HIF-1*, hypoxia-inducible factor 1, *CREB* cAMP response element binding, *PPAR-α* peroxisome proliferator-activated receptor-α, *PPAR-γ* peroxisome proliferator-activated receptor-γ, *P53* tumor protein 53, *PAF* platelet-activating factor; *OLDA* *N*-oleoyldopamine, *IGF-1* insulin-like growth factor-1, *CuZn-SOD* CuZn superoxide dismutase, *GSH-Px* glutathione peroxidase, *GluR6* glutamate receptor-6, *Glut-1* glucose transporter-1, *5-HT* 5-hydroxytryptamine, *GHB* γ-hydroxybutyric acid, *GABA* γ-aminobutyric acid, *IL-1ra* IL-1 receptor antagonist, *HPGDS* hematopoietic prostaglandin D synthase, *Ngb* neuroglobin, *HSP* heat shock protein, *NPD1* 10,17*S*-docosatriene/neuroprotectin D1, *UCP2* uncoupling protein 2, *NF-κB* nuclear factor-κB, *Bcl-2* B-cell lymphoma 2

Although lipid messengers and hormones gained more attention in neuroprotection, proteins were still the dominant innate protective factors. For example, intrinsic antiapoptotic factors such as Bcl-2 and BNDF have been shown to prevent death of neurons under ischemia [47]. 10,17*S*-docosatriene (neuroprotectin D1, or NPD1) is a newly discovered endogenous early survival protein activated by oxidative stress to counteract leukocyte infiltration and proinflammatory gene expression in brain during ischemia/reperfusion [48]. NPD1 has also been found to be up-regulated by antiapoptotic proteins Bcl-2 and Bcl-xL, decreased by proapoptotic Bax and Bad, and inhibit the expression of cytokine-mediated cyclooxygenase-2 (COX-2) [49]. Hematopoietic prostaglandin D synthase (HPGDS) played a neuroprotective role by suppressing the activation and infiltration of inflammatory cells [50]. Ischemic preconditioning up-regulated endogenous uncoupling protein 2 (UCP2), which was thought to decrease reactive oxygen species (ROS) production, and protect the hippocampus from ischemia/reperfusion injury [51]. Neuregulin-1 is a pleiotropic endogenous growth factor and reported to play a neuroprotective role in experimental models of cerebral ischemia [52].

Many transcriptional factors are activated in response to injury and may play a role in cell survival. The nuclear factor-κB (NF-κB) is a transcriptional factor responding to a variety of stimuli and has been considered a central mediator of innate immunity [53]. In general, activation of NF-κB has been shown to exert an antiapoptotic effect through the regulation of genes for prosurvival proteins, such as β-galactosidase [54]. It also participates in cell proapoptotic signaling and exerts a significant protective role in regulating the accumulation of p53, cytochrome *c* release, and activating caspase-3 [55]. The dual effects of NF-κB are dependent on the time of its activation. Early NF-κB activation was found to contribute to neonatal hypoxia–ischemic brain damage and late activation led to endogenous neuroprotection including up-regulation of antiapoptotic molecules [56]. Other studies have also shown that NF-κB-mediated late preconditioning offers neuroprotection against subsequent insults [57]. Therefore, NF-κB is a double-edged sword, playing complex regulatory roles during the process of deciding cell fate under preconditioning and ischemia.

Hypoxia-inducible factor 1α (HIF-1α) is a well-characterized transcriptional factor of hypoxic response in mammalian cells. Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase enzymes (PHD), acetylated by arrest-defective-1 (ARD1), and bound by the von Hippel-Lindau protein (pVHL), which is subsequently subjected to ubiquitination [58]. However, HIF-1α could escape degradation under hypoxic conditions as its proline is not hydroxylated. Subsequently, the stabilized HIF-1α is transported to the nucleus and forms a dimer with aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1β). The dimer recruits transcriptional factors, binding to hypoxia response elements (HRE) in the promoter or enhancer regions of target genes [59]. Although the exact mechanism remains poorly elucidated, HIF-1α is known to regulate expression of lots of hypoxia responsive genes, promoting cell survival via a number of pathways including expediting oxygen-delivery to oxygen deprived tissues like EPO, supporting the formation of new blood vessels like vascular endothelial growth factor (VEGF), and increasing glucose transport like glucose transporter-1 (Glut-1) [60]. Recent studies have confirmed that HIF-1α induces EPO release by astrocytes and acts as an essential mediator of neuroprotection. This indicated that there is an intrinsic astrocytic–neuronal signaling pathway activated in hypoxic/ischemic injury [61]. Moreover, EPO was confirmed to be an important neuroprotective proteins observed in mouse retina hypoxic preconditioning model [62].

In addition to transcriptional factors, some receptors such as glutamate receptor subunit, glutamate receptor-6 (GluR6), are also neuroprotective, having antitoxic and anti-inflammatory properties. Ischemia preconditioning against ischemic brain injury in rat hippocampus showed a down-regulation of the assembly of GluR6-PSD95-MLK3 and thus inhibited the activation of MLK3, MKK4/7, JNK, as well as the phosphorylation of c-Jun and the expression of FasL. Both GluR6 antisense oligodeoxynucleotides and agonists reversed the above effects, implicating that GluR6 subunit-containing kainite receptors also have a significant role in preconditioning-induced neuronal survival [63].

Endogenous protective proteins are constantly being investigated as potential therapy targets. In the following section, we focus on two newly identified neuroprotective proteins, namely, 14-3-3γ and neuroglobin (Ngb). 14-3-3γ is an isotype of the 14-3-3 protein family and is thought to have antiapoptosis functions with strong binding capacity and multiple regulatory functions. Ngb is a recent addition to the vertebrate globin family and is an important protein for the movement of oxygen throughout the brain. Both types of protein were initially found in neurons and were later confirmed by our laboratory that they are also expressed in astrocytes and with protective properties against ischemia insult [64, 65].

14-3-3γ

14-3-3 proteins were named by Moore and Perez in 1967, during a systematic classification of brain proteins based on the fraction number of diethylaminoethyl (DEAE)-cellulose chromatography and their position after subsequent starch-

gel electrophoresis [66]. So far, 14-3-3 proteins have been found in all eukaryotic organisms with highly conserved structures, and they exhibit the highest expression level in the brain, accounting for 1% of soluble proteins in the cytoplasmic compartment, plasma membrane, and intracellular organelles [67]. The seven known isotypes of 14-3-3 proteins are identified with Greek letters: β, ε, γ, η, σ, τ, and ξ. All of them have good binding capability because of their highly conserved structure and their ligand only requires three or four specific amino acids, including phosphorylation of serine or threonine residues in the target sequence. They are known to bind with signal pathway related kinases, receptor proteins, and enzymes. They also serve to alter receptor–ligand binding capabilities by translocation and change the catalytic activity or protect the ligand from dephosphorylation or hydrolysis. This plethora of interacting proteins binding allows 14-3-3 protein to perform vital roles in a great many of physiological processes. An increasing amount of evidence has demonstrated that 14-3-3 proteins are involved in cell growth, cell proliferation, cell metabolism, signal transduction, cell cycle control, apoptotic cell death, gene transcription, protein translocation, stress response, tumorigenesis, and so on. Among them, the important function we concerned is that the 14-3-3 protein plays an important role against apoptosis [68].

It has been shown that the intensity of neuronal necrosis stained with 14-3-3 protein was markedly higher in where suffered ischemic injury in rat brain cortex [69]. Increasing evidences indicated that 14-3-3 proteins may be important on regulating other molecules to promote cell survival in neuronal cell in vivo, including under ischemia [70]. 14-3-3γ is expressed predominantly in the brain. It was originally considered only to be expressed in neurons. However, our previous work has demonstrated an expression of 14-3-3γ in the primary culture of mouse cortical astrocytes [65]. Astrocytes are the most abundant glial cells in the brain. They are important in sustaining a stable microenvironment. As the major component of blood–brain barrier, they are probably the first candidates suffering ischemic attack during a stroke. Additionally, there are a growing number of reports suggesting that astrocytes have significant regulatory roles including active participants in promoting cell survival during and after the onset of stroke. We further elucidated the potential protective function of 14-3-3γ in astrocyte under ischemia injury [24, 71, 72].

We used our well-established in vitro ischemia model in which the anaerobic chamber could mimic stroke conditions for the study [73]. Northern and Western blot analysis demonstrated that 14-3-3γ mRNA and protein were up-regulated in primary culture of mouse cerebral cortical astrocytes [24]. Overexpression experiments and antisense oligonucleotide treatments showed that an increase in 14-3-3γ protein levels could promote cell survival, while decrease enhance apoptosis under ischemia (Fig. 2). The protective mechanism occurred when endogenous 14-3-3γ was bound to p-Bad and prevented Bad from entering mitochondria to initiate apoptosis [71]. This finding was consistent with other reports that 14-3-3 proteins could interact with p-Bad to inhibit Bad-mediated cell apoptosis [74, 75].

The increase in expression of 14-3-3γ protein would enhance its protective roles indicating the significance of 14-3-3γ protein against ischemic injury. Our latest study demonstrated that both Akt and MAPK pathways were activated in murine cortical astrocyte in primary cultures under ischemia. The up-regulation of 14-3-3γ induced by ischemia involved JNK pathway, which further leads to the nuclear translocation of p-c-Jun to activate the transcriptional factor AP-1 [72]. While in another study in PC12 cells, ERK1/2 and p38 MAPK instead of JNK were found to up-regulate 14-3-3 protein in a model of preconditioning with hydrogen peroxide [76]. These studies implied astrocytes and neurons having different signaling pathways being activated under ischemia in regulating the expression of 14-3-3γ. Further clarification of these might shed light to the difference in tolerance between astrocytes and neurons to ischemia injury.

Similarly, up-regulation of 14-3-3 was observed in human brain with ischemic attack. 14-3-3 protein was detected in cerebrospinal fluid (CSF) of patients suffering from brain injury. Now, it could be considered as a potential new biomarker for clinical detection of CNS injury and diseases. Immunostaining of samples from an autopsy of a human brain suffered from cerebrovascular ischemia demonstrated a dominant up-regulation of many 14-3-3 proteins (β, γ, ε, σ, τ) in astrocyte. This up-regulation of 14-3-3 proteins were mainly distributed in the infarct lesions and particularly abundant in infarcts at chronic stage [77]. In patients suffering from mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), 14-3-3γ and 14-3-3τ proteins were detected in CSF of patients with stroke-like episodes even before MRI showing new brain lesions [78]. Thus, the up-regulation of 14-3-3γ under ischemia was demonstrated in both in vivo and in vitro models including human brain.

In addition to 14-3-3γ, many other isoforms of 14-3-3 proteins are also shown to be altered in CNS suffered from ischemia damage or oxidative-related stresses. 14-3-3β and 14-3-3ξ were thought to be released from degenerating neurons and rose markedly in CSF following experimental brain injury [79], thus they could be considered as potential surrogate makers for neuroprotection. The level of 14-3-3 proteins increased more frequently in neurons near the ischemic core either with 14-3-3σ in the cytoplasm or 14-3-3β in the nucleus. While in astrocytes, 14-3-3σ and 14-3-

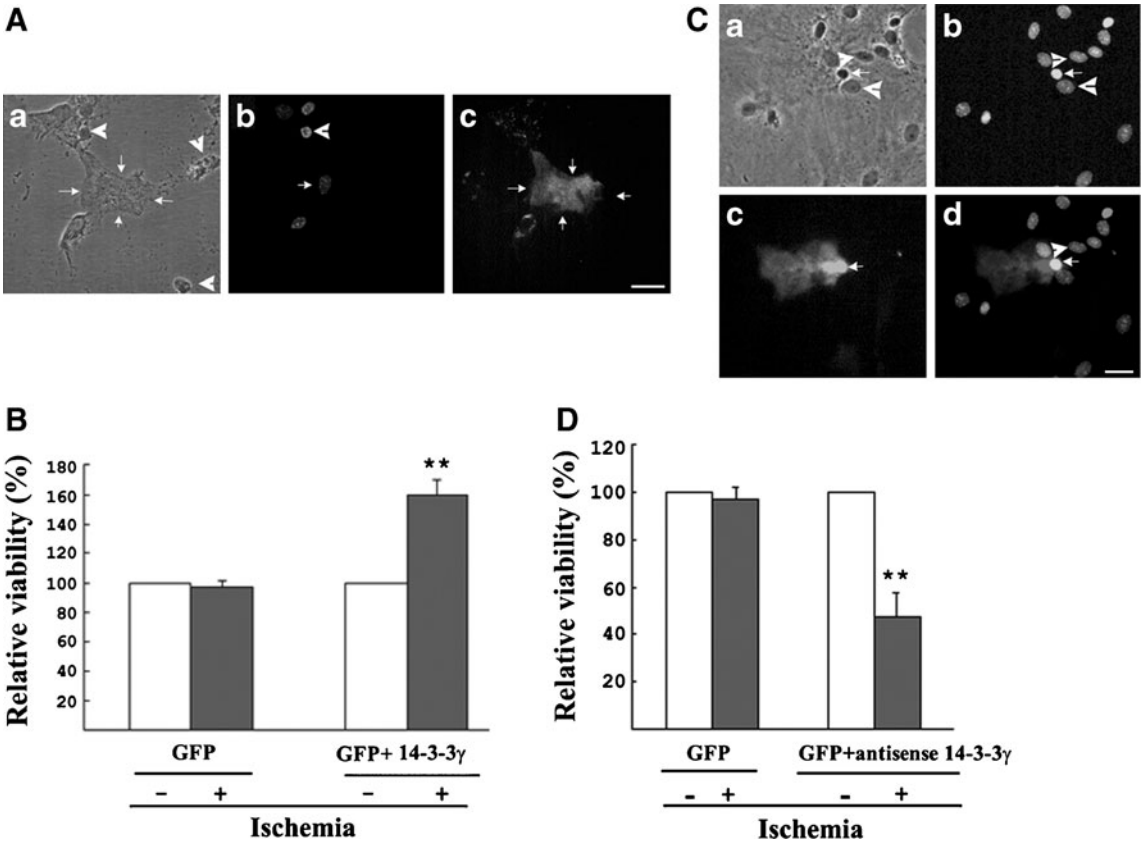

Fig. 2 14-3-3γ promotes cell survival in primary culture of astrocytes under ischemia. **a, b** Overexpression of 14-3-3γ in astrocytes enhanced viability under ischemia. **c**, **d** Astrocytes transfected with antisense 14-3-3γ contained lower level of 14-3-3γ and a higher cell death under ischemia. $^{**}p<0.01$. Adapted from Chen et al. [64]

3ε were positive in the cytoplasm, with only 14-3-3γ being positive in the nuclei [80]. In culture of human astrocytes, the expression of 14-3-3σ as well as p53 and p21 was up-regulated in astrocytes following exposure to hydrogen peroxide, 4-hydroxy-2-nonenal (4-HNE), or etoposide, a topoisomerase II inhibitor. 14-3-3σ was also induced by treatment with 5-aza-2′-deoxycytidine, suggesting a hypermethylated status of the gene promoter in astrocytes. In vivo, a small subset of hypertrophic reactive astrocytes expressed 14-3-3σ in active demyelinating lesions of multiple sclerosis and ischemic lesions of cerebral infarction. These observations suggest that 14-3-3σ might serve as a marker of oxidative and DNA-damaging stresses inducing the mitotic checkpoint dysfunction in reactive astrocytes under pathological conditions [81]. However, 14-3-3η was selectively up-regulated in neurons but down-regulated in astrocytes during in vitro development, and its gene expression did not show any changes by ischemia in neurons nor astrocytes [82]. Ligand-activated peroxisome proliferator-activated receptor-γ confers resistance to neuronal apoptosis and cerebral infarction by driving 14-3-3ε up-regulation, which plays a similar role as 14-3-3γ to enhance sequestration of phosphorylated Bad and thereby suppresses apoptosis [83]. Taken together, different isoforms of 14-3-3 protein were found highly expressed in brain damage including ischemia response for distinct functions. These 14-3-3 proteins, taken together with 14-3-3γ, are important in protecting the CNS at an early phase of injury. Therefore, it is worthwhile to elucidate their roles and mechanism of protection under CNS with special emphasis under ischemia.

Besides the protective functions observed in ischemia models, the protection of 14-3-3 proteins has also been noticed in preconditioning studies. Preconditioning could inhibit the phosphorylation of 14-3-3 proteins and prevent Bax translocation to mitochondria, and thus decreased the release of cytochrome-c and depressed caspase-3 activation through the GluR6-mediated signal pathway [63]. Apoptosis signal-regulating kinase 1 (ASK1) was found to bind to 14-3-3 protein under normal physiological conditions. Under preconditioning, the dissociation of ASK1 from 14-3-3 protein was suppressed and prevented apoptosis under ischemic preconditioning [84]. Research also demonstrated an elevation of 14-3-3 protein through the ERK1/2 signaling

pathways in an anoxic preconditioning experiment in neonatal rat cardiomyocytes responsible to the attenuation of myocardial injury caused by ischemia/reperfusion [85].

There is no doubt that endogenous 14-3-3 proteins are important intrinsic protective factors in resistance to ischemic damage and promotion of cell survival. The specific up-regulation and protection of 14-3-3γ in astrocyte by ischemia further reconfirmed its importance. Elucidation of its earlier induction mechanism would allow future development of pharmacotherapy to control its intrinsic levels and thus provide an important means in lengthen therapeutic time window for ischemia treatment.

Neuroglobin

Globins are porphyrin-containing proteins that bind oxygen in a reversible manner. Their primary functions are thought to be oxygen storage and transportation, facilitating movement into mitochondria, scavenging reactive oxygen species, and detoxifying nitric oxide. Globins have been found in many species, including bacteria, fungi, plants, and animals. There are four globins found in vertebrates, namely, hemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb), and cytoglobin (Cygb).

Ngb was recently discovered to exist primarily in the nervous system [86]. The human brain constitutes only 2% of the total body weight and yet utilizes 20% of the oxygen taken in by the lungs. Ngb has been shown to bind oxygen with higher affinity than Hb. Therefore, Ngb could serve to store and transport oxygen in the brain, analogous to Mb in muscle [87]. Mitochondria are critical energy supplies to support cell functions. Hence, Ngb is thought to facilitate oxygen movement into mitochondria, playing a critical role in the regulation of oxygen homeostasis in the CNS [88].

Similar to Mb, the core feature for Ngb is to regulate oxygen homeostasis in brain. This function suggests that Ngb has the potential to be a mediator of ischemic injury–repair coupling in the CNS. Cerebral ischemia usually results from the interruption of blood flow in cerebral circulation. The clinical implications of protective functions of Ngb have been investigated using in vivo ischemic models. In a rat focal ischemia model of middle cerebral artery occlusion (MCAO), cerebral infarct size was found to be increased upon administration of Ngb antisense oligodeoxynucleotide through the intracerebroventricular route and reduced after intracerebral administration of an adeno-associated virus vector with Ngb gene [89]. Similar findings were also reported in Ngb transgenic mice with overexpression of Ngb [90]. Moreover, an increase in expression of Ngb was observed in the cortical peri-infarct region of stroke patients. This further suggested that Ngb makes a novel and potential target for future stroke therapy [91].

Using in vitro primary cultures of cerebral cortical astrocytes and neurons, we have demonstrated the expression of Ngb not only in neurons, but also in astrocytes; Ngb antisense treatment reduced the Ngb level in astrocyte and at the same time enhanced apoptosis under ischemia [64] (Fig. 3). Our recent preliminary observation found the expression level of Ngb in neurons at a higher level and correlated to its developmental stage; however, the expression was at a lower level in astrocytes and unrelated to the age of astrocytes in culture (unpublished data). As stated above, Ngb was now widely accepted to play a critical role in regulating oxygen homeostasis in the CNS [88]. Therefore, based on these observations, it is reasonable to hypothesize that higher level of Ngb protein in neurons could create a concentration gradient in favor of the movement of oxygen from astrocytes to neurons. Furthermore, astrocytes form the blood–brain barrier and would be the first cell type in the CNS to encounter the oxygen transferred from the Hb in the blood. For this oxygen to be able to reach neurons, Ngb in astrocytes will play an indisputable role. There are indications that astrocytes are

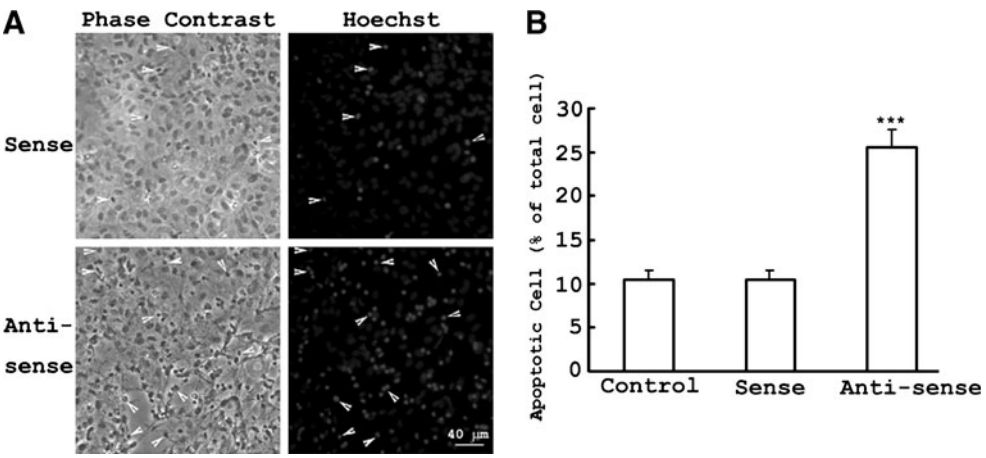

Fig. 3 Effect of Ngb sense and antisense treatments on ischemia-induced apoptosis in astrocytes. **a**. The number of apoptotic nuclei increased significantly in antisense-treated cultures. **b**. The estimated number of apoptotic cells significantly increased in cultures transfected with antisense Ngb as compared with the untransfected and sense-transfected controls. ***$p<0.001$. Adapted from Chen et al. [71]

potentially responsible for storing and supplying oxygen to benefit neurons when required, especially under hypoxic or ischemic conditions [92]. Despite the inelucidated mechanism, there is no doubt that Ngb processes the potential to play a protective role in astrocytes and neurons [93], especially during ischemia. Furthermore, Ngb may function as a NO-dioxygenase, an oxygen sensor or scavenger for reactive oxygen species, in a similar manner to the other types of globins [94].

Our unpublished preliminary findings suggest that Ngb may be colocalized and comigrated with mitochondria in neuronal processes [93]. Mitochondria were clustered in the neuronal soma but distributed sparsely in the processes. The motility behavior of mitochondria in nerve axons displayed salutatory and bidirectional movements through a combination of dynamic events [95].

There is no doubt that Ngb is the oxygen-binding protein expressed in both astrocytes and neurons. The up-regulation of Ngb in ischemic astrocytes and neurons can offer protection on neural cells during ischemia. Our unpublished observation on Ngb and mitochondria in astrocytes and neurons strongly suggested a close relationship of Ngb with mitochondria; the subcellular organelles utilize most of the oxygen in a cell. Investigation on the Ngb expression and protective mechanism would be important for the understanding of oxygen metabolism and transportation in the CNS. This information is especially essential for development efficient management and treatment of patients with stroke attack.

Conclusion

In this review, we have presented numerous studies describing various intrinsic factors with protective roles against ischemic insult. There is an increasing interest on clarifying how preconditioning triggers these intrinsic protective proteins. The discovery of 14-3-3γ and Ngb is a promising addition to the list. Interestingly, Ngb and 14-3-3γ were recently found to be closely interrelated to neuronal defensive machinery: silencing Ngb will increase the vulnerability to oxidative stress by down-regulating 14-3-3γ [96]. Understanding the roles of these intrinsic protective proteins and their relationship and interactive action may be important in elucidating how our innate protective systems manage to protect ourselves from injury. This would certainly provide us new insights on future development of various stroke therapies and management by fully utilizing new pharmacotherapy to arouse the endogenous protective mechanism. The arousal of these intrinsic protective mechanisms would be able to lengthen the therapeutic time window, and thus suitable treatments could be implicated in time for stroke patients to reduce mortality rate and improve treatment outcomes.

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