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Insights into cytoprotection from ground squirrel hibernation, a natural model of tolerance to profound brain oligaemia

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Abstract

Progression of acute ischaemic brain damage is complex and multifactorial. Also, evidence suggests that participating molecules and signal transduction pathways can function differently in different cellular contexts. Hibernation torpor, a model of natural tolerance to profoundly reduced blood flow and oxygen delivery to brain, along with models of induced ischaemic tolerance can guide efforts to identify cytoprotective mechanisms that are multifactorial and that target multiple mechanisms in multiple cellular contexts. Post-translational modification of proteins by conjugation with the SUMO (small ubiquitin-related modifier) is massively increased in hibernation and may be such a mechanism.

Keywords

Akt; cytoprotection; hibernation; ischaemia; oligaemia; small ubiquitin-related modifier (SUMO)

Introduction

Progression of cellular damage during acute brain ischaemia is multifactorial and complex [1]. Efforts to accurately model this process are hampered by the sheer number of participating molecular mechanisms and by their intricate interplay. For example, a list of general pathobiological mechanisms in acute brain ischaemia (each encompassing multiple molecular mechanisms) might include metabolic failure, membrane failure, excitotoxicity, apoptosis, oxidative/nitrative stress, pathological gene expression, neurovascular unit dysfunction and inflammatory/immune processes [2,3]. Within one of these general categories, inflammatory and immune responses, cytokines would be active players. More than 100 cytokines have been identified. One of these cytokines is TNF (tumour necrosis factor). TNF has a type I and a type II receptor [4]. The type I receptor activates the multifunctional transcription factor NF- κ B (nuclear factor κ B), which regulates the expression of over 150 target genes [5]. A study mapping the protein interaction network forming the link between the TNF-activated type I TNF receptor and NF- κ B identified 221 molecular associations [6]. *In toto*, this level of complexity is extraordinarily difficult to incorporate into high-fidelity models of progressive injury in acute brain ischaemia. The difficulty in generating high-fidelity ischaemic injury models that reflect the interacting mechanisms of the full biological system may contribute to selection of ineffectual targets for clinical stroke therapy trials.

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Tolerance models

Faced with this level of complexity, investigators in many laboratories have turned to examination of endogenous cytoprotective mechanisms that can be induced *in vitro* in cultured cells or *in vivo* in animal stroke models by PC (preconditioning) with various forms of sublethal stress [7]. The aim is to identify and target the mechanisms that confer protection in these states of ischaemia tolerance.

There are also natural states that confer robust resistance to severe reductions in blood flow and capacity to deliver oxygen. One such state is hibernation. Hibernation in mammals has evolved in at least six mammalian orders as an adaptation to noxious environmental conditions [8]. Hibernation torpor in 13-lined ground squirrels (*Spermophilus tridecemlineatus*) is characterized by profound reductions in heart rate (e.g. 400–20 beats/min), cerebral blood flow [e.g. 65–7 ml · (100 g)⁻¹ · min⁻¹] [9] and cerebral glucose utilization [CMR_{Glc} (cerebral metabolic rate for glucose) (e.g. 0.80–0.01 μmol · min⁻¹ · g⁻¹) [10] with body temperatures slightly above ambient temperature (e.g. 0–5°C). During hibernation torpor, ground squirrels can tolerate ‘trickle’ blood flow and a severely reduced capacity to deliver oxygen for many weeks at a time and recover without any evidence of cellular damage in the brain [9]. This prolonged tolerance of severe oligoemia does not simply represent preservation of homeostasis under extreme conditions. Hibernation has been shown to reflect a state in which neuroprotection from a superimposed lethal ischaemic insult exists. Hippocampal slices from hibernating ground squirrels have increased resistance to CA1 pyramidal cell death from oxygen/glucose deprivation at 36, 20 and 7°C compared with slices from non-hibernating ground squirrels and rats [11]. Also, the injury response of brain tissue to insertion of microdialysis probes is markedly attenuated during hibernation [12].

Apparent dependence of Akt on cellular context

We have examined the serine–threonine protein kinase, Akt, signal transduction pathway (shown to be cytoprotective in many models) in ground squirrels during hibernation torpor [13]. Akt can counter apoptosis by phosphorylating and inactivating proteins that can activate apoptotic pathways such as the Bcl-2 (B-cell lymphocytic-leukaemia proto-oncogene 2) family member, BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death), procaspase 9, GSK3 (glycogen synthase kinase 3) and FOXO (forkhead box O) family proteins. Direct targets of Akt also include some ubiquitin ligase proteins and MDM2 (murine double minute 2), which is responsible for the negative regulation of p53, a known pro-apoptotic transcription factor. The activation of Akt is dependent on its translocation to the plasma membrane and its association via the pleckstrin homology domain with the phospholipid, PtdIns(3,4,5)P₃, a product of the conversion by PI3K (phosphoinositide 3-kinase) of PtdIns(4,5)P₂. Phosphorylation on Thr³⁰⁸ and Ser⁴⁷³ occurs following Akt translocation to the plasma membrane and is required for enzymatic activation of that signal transduction molecule [14].

Hibernation torpor is associated with down-regulation of Akt. During ground squirrel hibernation torpor, levels of Akt phosphorylation are significantly decreased in brain and muscle. Similar decreases in liver, heart and kidney (Ser⁴⁷³)-phospho-Akt indicate that the suppression of Akt phosphorylation on this residue is generalized. Total Akt levels in these tissues remain unchanged in this state. The hypophosphorylation of Akt noted during hibernation corresponds to a reduction of brain Akt kinase activity to approximately one-half the level seen in active ground squirrels. This decrease in activity appears paradoxical because, as stated above, a decrease in Akt activity would be expected to be pro-apoptotic, but hibernation torpor is a stress-tolerant state.

In view of this apparent discrepancy and in order to further our understanding of signalling cascades involved in the phenomenon of tolerance, we developed a model of ischaemic

tolerance in PC12 cells [15]. We demonstrated that OGD (oxygen and glucose deprivation) induces cell death in PC12 cells and that 6 h of OGD PC counteracts cell death otherwise induced by 15 h of OGD 1 day later. Using our model of ischaemic tolerance in PC12 cells, we addressed whether PC to induce ischaemic tolerance also down-regulates Akt in PC12 cells. Our kinetic studies during the first 6 h of the 15 h severe OGD showed that phosphorylation of Akt did initially increase on Western blots with exposure to OGD, but that this increase was attenuated in tolerized cells [16]. We then examined in similar kinetic studies whether lower Akt phosphorylation levels correlate with a reduction in Akt activity; we measured phosphorylation levels of several important downstream Akt targets in preconditioned compared with naïve PC12 cells. We found a significant decrease in phosphorylation levels of the protein kinase, GSK3, and the FOXO family transcription factor, FOXO4, in tolerized cells. Down-regulation of Akt activity was further confirmed by examining levels of phosphorylated MDM2, another substrate of Akt. Compared with naïve cells, cells preconditioned 24 h earlier exhibited a lower OGD-induced increase in phosphorylated MDM2. In addition, pharmacological blockade of the PI3K/Akt pathway with a PI3K inhibitor (LY294002) reduced OGD-induced cell death and increased the protective effect of PC. Decreasing the availability of phospho-Akt by transfecting the PC12 cells with inactive Akt constructs (T308A and/or S473A) also resulted in protection against OGD and potentiation of the protective effect of PC [16].

PC-induced tolerance that increases cell survival would be expected to correspond to greater rather than lesser Akt activation. However, in the PC12 cell model, a lesser Akt activation during the first 6 h of OGD is associated with a reduction in cell death, indicating that in the context of tolerance, modulation rather than full activation of Akt can be beneficial. A possible explanation for this apparent discrepancy is that besides their well-documented role in apoptosis, GSK3 and p53, as well as the FOXO superfamily of transcription factors, exert an essential regulatory influence on key cellular functions such as cell proliferation and growth arrest through their multiple targets [17–20]. Depending on the cell context, these proteins can trigger apoptotic responses or cell-cycle arrest; under conditions of reduced energy, these proteins can drive cells into a state of quiescence that might be neuroprotective. In fact, recent work has shown that FOXO protein family members are essential for the long-term survival of quiescent cells and that these proteins play an important role in cellcycle arrest [21]. Previously, Imae et al. [22] reported that FOXO protein levels were increased by fasting, suggesting that under conditions of metabolic stress, activation of FOXO transcription factors may be essential for survival. In the nematode *Caenorhabditis elegans*, it is necessary to activate DAF-16 (decay-accelerating factor 16), a forkhead transcription factor homologous with members of the mammalian FOXO family, to induce formation of the stress-resistant dauer larva stage under conditions of starvation and crowding [23].

SUMO (small ubiquitin-related modifier)

Given the multifactoriality and complex interactions of cellular injury mechanisms in acute brain ischaemia, cytoprotective mechanisms that are multifunctional and target multiple mechanisms in multiple cellular contexts are particularly attractive. The post-translational modifier, SUMO, affects proteins involved in gene expression, chromatin structure, signal transduction and maintenance of the genome (reviewed in [24]). The major targets for SUMO conjugation are transcription factors, and SUMOylation of these proteins mainly produces negative effects on gene expression. This prompted us to determine initially whether SUMO conjugation status changes during the metabolic rate suppression of ground squirrel hibernation torpor.

We found on Western blotting that there is a marked elevation of protein SUMOylation in most areas of the brain during the torpor phase of hibernation, with a concomitant loss of free SUMO

[25]. SUMO conjugation was noted to decrease quickly on emergence from torpor, indicating that SUMO conjugation and hibernation torpor are tightly connected. Although free SUMO is distributed throughout the cell, most of the conjugated SUMO is nuclear, consistent with transcription factors being major targets of SUMOylation [26].

Hibernation torpor-associated SUMOylation is not, however, restricted to the brain: an even greater SUMO conjugation occurs in liver and kidney tissue. Unlike other organs we examined, spleens were heavily SUMOylated even in active animals. Since other analyses of SUMO tissue distribution have been minimal, it is unclear whether these tissue distributions are typical of most vertebrate tissues or are restricted to hibernating animals.

The SUMOylation cycle involves at least four enzymes in a multistep process. The E1-activating enzyme ubiquitin-associated protein 2/activation of Smt3p1 initiates the first step. Then, the SUMO-specific E2-conjugating enzyme, Ubc9 (ubiquitin-conjugating enzyme-9), receives the activated SUMO and transfers it to conjugate with a substrate protein, sometimes with the help of an E3-ligase. A group of isopeptidases [SENPs (sentrin-specific proteases)] that deconjugate SUMOylated proteins complete the cycle [24]. We find that the expression levels of Ubc9, the single E2-conjugating enzyme in the SUMO pathway, are closely correlated with SUMO conjugation levels both in the brain and the kidney during hibernation.

We also examined the cytoprotective properties of SUMO-ylation in SHSY5Y cell cultures. By means of transfected wild-type or dominant-negative constructs, Ubc9 expression levels were shown to be critically related to the capacity of SHSY5Y cells to survive ischaemic insults (Table 1). Ubc9 is reported to be essential for viability of higher eukaryotic cells; Ubc9-deficient mouse embryos die at the early post-implantation stage [27], and depletion of Ubc9 in chicken DT40 cells results in death by apoptosis [28].

In summary, strong activation of Akt has been demonstrated to be cytoprotective in many models of cellular stress involving apoptotic stimuli, DNA-damaging agents and toxins. But strong inhibition of Akt has been demonstrated by genetic and biochemical analyses to activate the FOXO orthologue, DAF-16, and to induce dauer larval formation in *C. elegans*, a state that permits these organisms to withstand food deprivation and temperature stress [29]. Further, we have shown both that mammalian hibernation torpor in *S. tridecemlineatus* is associated with modulated suppression of Akt function and that PC12 cells tolerized by PC modulate the activation of Akt and resist damage in subsequent OGD exposures. Akt, therefore, under different conditions, can apparently be optimally cytoprotective when it is fully activated, when it is inactivated or if it undergoes modulated activation or modulated inactivation depending on the set or context of the cells. This serves to emphasize the need to understand the influence of the biological system (that constitutes the set or context of the cell) on a signal transduction pathway under any given condition in order to accurately predict the effects of activity changes in a key component of that pathway.

SUMOylation serves as a multifunctional regulator that becomes massively increased in ground squirrel hibernation torpor and provides impressive cytoprotection in SHSY5Y cell OGD. PC initially increases SUMOylation levels that return to baseline levels by 24 h after PC. However, preconditioned cells maintain normal protein SUMOylation levels during severe OGD, in contrast with a 50% decline in non-preconditioned cells. Suppression of the capacity to SUMOylate proteins is cytotoxic and nullifies cytoprotection by PC with or without hypothermia in these cells [25]. Further work will determine whether maintenance or elevation of SUMO conjugation levels is an appropriate target for drug discovery in the cerebrovascular disease field.

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Abbreviations used

DAF-16

decay-accelerating factor 16

FOXO

	forkhead box O
GSK3	glycogen synthase kinase 3
MDM2	murine double minute 2
NF-κB	nuclear factor κ B
OGD	oxygen and glucose deprivation
PC	preconditioning
PI3K	phosphoinositide 3-kinase
SUMO	small ubiquitin-related modifier
TNF	tumour necrosis factor

Table 1

Overexpression of Ubc9 wild-type increases and overexpression of Ubc9 DN (dominant-negative) decreases cell survival during OGD (12 h)

Results are mean \pm S.D. percentages of cell survival for three experiments. PC, PC (6 h OGD).

Cell type	Without PC	PC at 37°C	PC at 4°C
Control	45 \pm 6.5	60 \pm 9.1	66 \pm 6.7
Ubc9 (wild-type)	65 \pm 5.0	72 \pm 2.6	76 \pm 4.0
Ubc9 (DN)	21 \pm 8.1	25 \pm 5.2	25 \pm 5.0