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Is age a key factor contributing to the disparity between success of neuroprotective strategies in young animals and limited success in elderly stroke patients? Focus on protein homeostasis

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Abstract

Neuroprotection strategies to improve stroke outcome have been successful in the laboratory but not in clinical stroke trials, and thus have come under scrutiny by the medical community. Experimental stroke investigators are therefore under increased pressure to resolve this problem. Acute ischemic stroke represents a severe form of metabolic stress that activates many pathological processes and thereby impairs cellular functions. Traditionally, neuroprotection strategies were designed to improve stroke outcome by interfering with pathological processes triggered by ischemia. However, stroke outcome is also dependent on the brain's capacity to restore cellular functions impaired by ischemia, and this capacity declines with age. It is, therefore, conceivable that this age-dependent decline in the brain's self-healing capacity contributes to the disparity between the success of neuroprotective strategies in young animals, and limited success in elderly stroke patients. Here, prosurvival pathways that restore protein homeostasis impaired by ischemic stress should be considered, because their capacity decreases with increasing age, and maintenance of proteome fidelity is pivotal for cell survival. Boosting such prosurvival pathways pharmacologically to restore protein homeostasis and, thereby, cellular functions impaired by ischemic stress is expected to counterbalance the compromised self-healing capacity of aged brains and thereby help to improve stroke outcome.

Stroke is a serious medical condition that affects about 800,000 in the US every year.[1] Brain damage after stroke can cause devastating loss of brain functions and disability, creating a major burden for patients and their families as well as public and private health care systems. Therefore, developing treatment strategies that minimize brain damage after stroke is a high priority.

The pathophysiology of acute ischemic stroke has been investigated extensively in animal models, and strategies have been developed to improve stroke outcome by blocking pathological processes triggered by ischemia, including excitotoxicity, oxidative stress, and inflammation.[2–9] Such neuroprotection strategies have been effective in experimental stroke studies, but have largely failed in clinical trials. Many factors that

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potentially contribute to this disparity in outcomes have been identified,[2,10–18] including lack of rigor in design and execution of preclinical studies, use of animal stroke models that do not mimic stroke in patient regarding pathophysiology, and use of healthy animals for experimental studies that do not exhibit comorbidities typical for stroke patients. Strategies to remove such barriers in translational stroke research have been extensively discussed and suggested to be considered for future experimental stroke studies.[19–25] Age has attracted less attention as a factor potentially contributing to unsuccessful translational stroke research, even though the neuroprotection strategies that failed in clinical trials on elderly stroke patients were developed in experimental stroke studies performed primarily in young animals. Notably, the contribution of age has been considered predominantly with respect to co-morbidities characteristic of stroke patients. However, aging is also associated with a marked decline in the brain's self-healing capacity to restore cellular functions impaired by ischemic stress.[26–32]

Age is a key risk factor for ischemic stroke. In acute ischemic stroke, the ischemic core is surrounded by the penumbra—brain tissue that is metabolically compromised but still viable.[33] The penumbra is, therefore, a primary target for acute stroke therapies. Imaging technology is used in the clinic to measure the size of the penumbra,[34–39] in order to assess the potential benefits of therapeutic interventions to block the expansion of the ischemic core into the penumbra. In preclinical studies, a battery of imaging approaches is at hand to evaluate the size of the penumbra with great precision, including autoradiography, metabolic imaging, and high-resolution MRI. Imaging technologies used in the clinic, including MRI, PET, and CT, also provide sufficient resolution to evaluate the size of the penumbra and thereby assess the potential benefits of therapeutic interventions. Notably, the risk for potentially salvageable penumbra to convert to infarcted tissue increases with age,[40] and functional recovery from stroke decreases with age,[32,41] suggesting that aging is associated with a decline in the brain's self-healing capacity to restore cellular functions impaired by ischemic stress. This could have a major implication on outcomes of clinical stroke trials that test neuroprotection strategies that were developed in young animals. Indeed, such strategies are based on the assumption that stroke outcome will be improved by blocking ischemia-induced pathological processes as the cellular capacity to restore functions after ischemia is not compromised by aging. It is, therefore, predictable that such neuroprotection strategies will be less effective in the elderly patients after acute ischemic stroke because of the age-related decline in the brain's self-healing capacity.

In our search for novel approaches to improve stroke outcome in the clinical setting, we thus began to focus on processes that play key roles in maintaining cellular homeostasis, but that decline with age and are impaired by ischemic stress. Here, a promising target is protein homeostasis (proteostasis), that is, the dynamic equilibrium between protein synthesis and maturation, keeping proteins functional, and degradation of proteins by the ubiquitin/proteasome system or by autophagy. Proteostasis is achieved by many proteins acting in concert to maintain proteome fidelity in the cells, and notably, the proteostasis maintenance capacity declines with increasing age.[42,43] Indeed, impaired proteostasis is associated with a variety of age-related diseases.[44,45] In postmitotic cells such as neurons and cardiomyocytes, loss of proteostasis is of particular concern because damaged proteins can accumulate over time and form potentially toxic aggregates. Proteostasis is maintained by a complex network involving about 1400 proteins in human cells.[46] Of note, proteins are required for all cellular processes and the capacity to maintain proteostasis declines with age.[42] Further, the signaling pathways that regulate longevity modulate the capacity of cells to maintain proteostasis, indicating the critical importance of functional proteins to keep cells viable. Together, this suggests that at advanced age all cellular processes are at risk in pathological conditions associated with impaired proteostasis, including ischemic stroke.

A network of cellular processes maintains proteostasis by ensuring that newly synthesized proteins are functional and that unfolded or misfolded proteins are cleared from cells, and restores proteostasis impaired by stress. Three of these processes are important to discuss here specifically: small ubiquitin-like modifier (SUMO) and ubiquitin conjugation, autophagy, and the unfolded protein response (UPR). SUMO and ubiquitin conjugation are key components of the protein quality control system that ensure that newly synthesized proteins are functional and that unfolded or misfolded proteins are cleared from cells through the ubiquitin-proteasome system.[47–50]

Autophagy is activated under cellular stress conditions to restore protein homeostasis by lysosome-mediated degradation of large protein aggregates.[51] Autophagy has been implicated in many human diseases, and its activation is regarded as a protective mechanism that allows cells to survive under stress conditions.[52,53] The autophagy pathway is activated in brain ischemia/stroke, and reports from experimental studies demonstrate that activation of autophagy is a neuroprotective stress response.[54–57] Notably, autophagy activity decreases with increasing age.[58] It is, therefore, highly likely that the age-related decline in autophagy activity compromises the cellular capacity

to restore proteostasis impaired by stress, and contribute to worse stroke outcome in aged brains.

UPR is activated in response to accumulation of unfolded/misfolded proteins in the lumen of the endoplasmic reticulum (ER), the cellular organelle in which newly synthesized membrane and secretory proteins are folded and processed.[59] The UPR has three response branches that are controlled by stress sensor proteins located in the ER membrane—the activating transcription factor 6 (ATF6), the inositol-requiring enzyme-1 (IRE1), and the protein kinase RNA-like ER kinase (PERK).[59,60] The IRE1 UPR branch is of particular significance here because it plays a prominent role in restoring proteostasis impaired by stress. ER stress-activated IRE1 triggers splicing of X-box binding protein-1 (*Xbp1*) mRNA which causes a frame shift of the coding sequence and consequent formation of the new protein spliced XBP1 (XBP1s). XBP1s is a transcription factor and regulates expression of subsets of genes that code for ER-resident chaperones and enzymes of the hexosamine biosynthetic pathway (HBP).[61–63] HBP produces uridine diphosphate N-acetylglucosamine, the substrate for O-linked β-N-acetylglucosamine (O-GlcNAc) modification of proteins (O-GlcNAcylation). Notably, increased O-GlcNAcylation protects cells against injury associated with a variety of ER stress-related conditions.[64–67] Studies on *Caenorhabditis elegans*, a widely used model system for aging research, suggest that the IRE1 UPR branch is a key component of the network of cellular processes involved in restoring proteostasis impaired by stress.[68,69] Specifically, overexpression of XBP1s in neurons and O-GlcNAcylation of proteins involved in gene expression confer protection against stress conditions that impair proteostasis. Further, both XBP1s overexpression and O-GlcNAc cycling at genes positively correlate with longevity, which highlights the importance of proteostasis in aging cells, and suggests that restoration of proteostasis impaired by stress is critical to the survival of cells compromised by disease associated with aging, such as stroke. Together, these observations suggest that activation of the IRE1/XBP1/HBP/O-GlcNAc axis plays a key role in helping cells to withstand stress conditions that impair proteostasis.

Results from a variety of experimental studies support the notion that transient brain ischemia impairs proteostasis, as evidenced by suppression of protein synthesis, formation of protein aggregates, and activation of ubiquitin and SUMO conjugation and UPR.[26,60,70–74] Importantly, ubiquitin and SUMO conjugation and O-GlcNAcylation are markedly increased in brains of young mice exposed to a short ischemic stress, but this activation is severely impaired in aged brains.[26] This suggests an aging-related decline in the brain's capacity to restore proteostasis impaired by ischemic stress. In acute ischemic stroke, the XBP1-induced O-GlcNAcylation of proteins (IRE1/XBP1/HBP/O-GlcNAc axis) plays a key role in defining stroke outcome in mice.[75] Specifically, deletion of XBP1 in neurons worsens stroke outcome; stroke activates O-GlcNAcylation in neurons of the penumbra that is XBP1-dependent and pharmacologic boosting of O-GlcNAcylation improves stroke outcome. These findings suggest that restoration of proteostasis critically defines stroke outcome and that activation of XBP1-induced O-GlcNAcylation plays an essential role in neuroprotection in experimental stroke. Interestingly, restoration of proteostasis is also an important component of the neuroprotective effects of preconditioning that helps neurons to better withstand stress conditions,[76] and further, aging has been associated with reduced neuroprotective effects of preconditioning in both preclinical and clinical studies.[77–79] Considering the potential role of the IRE1/XBP1/HBP/O-GlcNAc axis in stroke outcome, it is plausible to expect that any aging-related impairment of this axis would have a detrimental effect. This is indeed the case. Stroke-induced activation of the IRE1/XBP1/HBP/O-GlcNAc axis in neurons of the stroke penumbra is severely impaired in aged brains, and this impairment is linked to worse stroke outcome.[75] Thus, experimental stroke studies in young rodents do not mimic the aging-related pathologic milieu associated with stroke in aged brains.

Whether the IRE1/XBP1/HBP/O-GlcNAc axis is involved in the outcome of patients suffering from ischemic stroke still needs to be verified. Considering that this pathway is highly conserved, it is very likely that the response of neurons to ischemic stress is not different in mice and humans. It will be challenging to investigate O-GlcNAcylation in brains of stroke patients because this is a highly dynamic protein modification that cannot be evaluated in postmortem brains. However, there is ample evidence for a pivotal role of the XBP1/HBP/O-GlcNAc axis in keeping neurons healthy, as a -116C/G polymorphism in the promoter region of *XBP1*, that results in impaired *XBP1* expression upon endoplasmic reticulum stress,[80] is associated with risk of Alzheimer's disease and ischemic stroke.[81,82] Furthermore, a variety of experimental studies reported a key role of the XBP1/HBP/O-GlcNAc axis in physiological and pathological states of the brain. Specifically, this axis is involved in memory processes,[83,84] caloric restriction improves memory deficit associated with diabetes by increasing levels of O-GlcNAc modified proteins in the hippocampus,[85] and loss of the O-GlcNAc transferase in excitatory neurons induces neurodegeneration,[86] while increasing O-GlcNAcylation slows neurodegeneration.[87]

The mechanisms underlying neuroprotection provided by activation of the IRE1/XBP1/HBP/O-GlcNAc axis are still not well understood. Much of

our understanding of how O-GlcNAcylation increases the resistance of cells to stress comes from experiments in cardiomyocytes and myocardial ischemia studies.[66,88–92] Specifically, O-GlcNAcylation attenuates calcium overload and reactive oxygen species (ROS) generation,[92] and ER stress-induced cardiomyocyte death,[66] increases mitochondrial Bcl-2,[93] and protects mitochondria from loss of membrane potential and formation of the permeability transition pore.[94] Notably, O-GlcNAcylation is also associated with isoflurane-induced cardioprotection.[91]

Solving the translational challenges of developing interventions that protect against ischemia-induced injury is likely a problem in many areas of medicine. Cardiac disease, for example, also has well defined aging-related components including impaired proteostasis.[95] Notably, age is a major risk factor for developing ischemic heart disease; ischemic tolerance is impaired in aged murine and human hearts, and the protective effects of ischemic preconditioning is lost in elderly patients.[96–98] Interestingly, this age-related decline in the effect of ischemic preconditioning to protect the heart from ischemic stress is rescued by physical activity and caloric restriction,[99] known to improve the capacity to maintain proteostasis with increasing age.[42] Despite the many cardioprotection strategies that have been developed and successfully tested in experimental studies, outcomes of clinical trials have been largely disappointing, and as in stroke research, strategies to increase the rigor of preclinical studies have been discussed and implemented in an effort to improve bench-to-bedside translational research.[100]

In conclusion, the age-related decline in the capacity to maintain proteostasis and to activate prosurvival pathways to restore cellular function impaired by ischemic stress could be a major factor contributing to ischemic stroke outcome in the clinical setting. Consequently, neuroprotection strategies that block ischemia-induced pathological processes, and thereby improve stroke outcome in preclinical studies on young animals, are likely to have limited success rates in elderly stroke patients. Therefore, future experimental stroke studies need to take into account the aging-related decline in the brain's self-healing capacity and include experiments on aged animals into the repertoire of preclinical stroke studies. Pharmacologic interventions could help to rescue the aging-related decline in the brain's self-healing capacity and thereby improve stroke outcome. Ultimately, a combination approach designed to block pathological processes and boost prosurvival pathways could be considered to be applied at an early time after stroke onset and thereby help cells in the stroke penumbra to withstand ischemic stress until blood flow can be restored in the clinic by more invasive interventions.

Declaration of conflicting interests

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