New strategies for Alzheimer disease and cognitive impairment

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Introduction

Alzheimer disease, cognitive loss, novel cellular pathways. For the population in the United States, the National Institute on Aging estimates that almost five million people have Alzheimer’s disease (AD). Furthermore, more than twenty-four million people suffer from AD, pre-senile dementia, and other disorders of cognitive loss worldwide. If one then includes other related degenerative disorders of the central nervous system (CNS), the scope of these illnesses approach 370 million people throughout the globe. With these disorders of cognition, the cost of physician services, hospital and nursing home care, and medications continues to rise dramatically. In addition, the medical costs parallel a progressive loss of economic productivity with rising morbidity and mortality, ultimately resulting in an annual deficit to the economy that is greater than $400 billion. Interestingly, the most significant portion of this economic loss is composed of only a few neurodegenerative disease entities, such as ischemic disease and AD. The annual cost per patient with AD is estimated at greater than $174,000 with an annual population aggregate cost of $100 billion.1,2

Despite the current understanding of the cellular pathways that modulate CNS injury during AD and cognitive disorders, complete therapeutic prevention or reversal of neurovascular injury during AD or dementia is not achievable. As a result, identification of novel therapeutic targets for the treatment of neurovascular injury would be extremely beneficial to reduce or eliminate disability from diseases that lead to cognitive loss or impairment. Here we describe the capacity of intrinsic cellular mechanisms for the novel pathways of erythropoietin and forkhead transcription factors that may offer not only new strategies for disorders such as AD and cognitive loss, but also function as biomarkers for disease onset and progression.

Oxidative stress and neurovascular injury. Release of reactive oxygen species (ROS) that consist of oxygen free radicals and other chemical entities can result in the development of oxidative stress in the body. Oxygen free radicals can be generated in elevated quantities during the reduction of oxygen and lead to cell injury. ROS can involve superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide (NO) and peroxynitrite.3-5 Most species are produced at low levels during normal physiological conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase (SOD), glutathione peroxidase, catalase and small molecule substances such as vitamins C and E. Other closely linked pathways to oxidative stress in conjunction with processes of mitochondrial dysfunction7-19 can contribute to a variety of disease states such as diabetes, ischemia, cognitive loss, Alzheimer’s disease and trauma.1,20-23 Oxidative stress can lead to apoptosis in neurons, endothelial
cells (ECs), cardiomyocytes and smooth muscle cells that involve separate as well as overlapping pathways.\textsuperscript{21,24-28}

Apoptosis is a dynamic process that consists of both the early exposure of membrane phosphatidylserine (PS) residues and the late destruction of genomic DNA.\textsuperscript{29,30} Externalization of membrane PS residues is an early event during cell apoptosis\textsuperscript{31,32} and can become a signal for the phagocytosis of cells.\textsuperscript{16,33,34} The loss of membrane phospholipid asymmetry leads to the exposure of membrane PS residues on the cell surface and assists microglia to target cells for phagocytosis.\textsuperscript{13,26,35-37} This process occurs with the expression of the phosphatidylserine receptor (PSR) on microglia during oxidative stress.\textsuperscript{38,39} It has been shown that blockade of PSR function in microglia prevents the activation of microglia.\textsuperscript{36,40} Externalization of membrane PS residues occurs in neurons, vascular cells and inflammatory microglia in conjunction with AD and cognitive loss during reduced oxygen exposure.\textsuperscript{6,41-44} β-amyloid (Aβ) exposure\textsuperscript{35,46} during AD progression, nitric oxide exposure,\textsuperscript{47-51} and the administration of agents that induce the production of ROS, such as 6-hydroxydopamine.\textsuperscript{52} Membrane PS externalization on platelets also has been associated with clot formation in the vascular system.\textsuperscript{53}

The cleavage of genomic DNA into fragments\textsuperscript{43,54,55} usually occurs after membrane PS exposure\textsuperscript{56} and is considered to be a later event during apoptotic injury.\textsuperscript{6,55,57,58} Several enzymes responsible for DNA degradation include the acidic, cation independent endonuclease (DNase II), cyclophilins, and the 97 kDa magnesium—dependent endonuclease.\textsuperscript{3,59} Three separate endonuclease activities also have been found in neurons that include a constitutive acidic cation-independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium dependent endonuclease.\textsuperscript{60,61}

During oxidative stress, mitochondrial membrane transition pore permeability also is increased,\textsuperscript{12,26,62,63} a significant loss of mitochondrial NAD\textsuperscript{+} stores occurs, and further generation of superoxide radicals leads to cell injury.\textsuperscript{3,64} Mitochondria are a significant source of superoxide radicals that are associated with oxidative stress.\textsuperscript{3,65} Blockade of the electron transfer chain at the flavin mononucleotide group of complex I or at the ubiquinone site of complex III results in the active generation of free radicals which can impair mitochondrial electron transport and enhance free radical production.\textsuperscript{39,59} Furthermore, mutations in the mitochondrial genome have been associated with the potential development of a host of disorders, such as hypertension, hypercholesterolemia and hypomagnesemia.\textsuperscript{66,67} ROS also may lead to cellular acidosis and subsequent mitochondrial failure.\textsuperscript{20} Disorders, such as hypoxia,\textsuperscript{68} diabetes\textsuperscript{69,70} and excessive free radical production\textsuperscript{60,71,72} can result in the disturbance of intracellular pH.

**Erythropoietin (EPO) and its Receptor**

**EPO and the EPO receptor.** The EPO gene is located on chromosome 7, exists as a single copy in a 5.4 kb region of the genomic DNA, and encodes a polypeptide chain containing 193 amino acids. During the production and secretion of EPO, a circulatory mature protein of 165 amino acids is produced.\textsuperscript{73,74} The principal organs of EPO production and secretion are the kidney, liver, brain and uterus. EPO production and secretion occurs foremost in the kidney.\textsuperscript{75}

Interestingly, increased levels of EPO in the fetal plasma and amniotic fluid during gestation may function as a biomarker of intravertine hypoxia.\textsuperscript{76} For biological systems, a “biomarker” can consist of any entity that occurs in the body and that can be measured to predict the diagnosis, onset or progression of a disease process.\textsuperscript{77} Novel pathways that involve the cytokine and growth factor EPO may indicate that the increased presence of this agent during periods of oxidative stress may lead to cellular mechanisms to protect against ROS.\textsuperscript{74,78,79} Recent studies have demonstrated that EPO is not only required for erythropoiesis, but also functions in other organs and tissues, such as the brain, heart and vascular system that can be relevant for the treatment of AD\textsuperscript{80-84} (Fig. 1). EPO production is believed to occur throughout the body,\textsuperscript{5,78,85} and can be detected in the breath of healthy individuals.\textsuperscript{86} In addition, it has been suggested that EPO may provide developmental cognitive support. In experimental animal models, EPO may reduce apoptotic pathways during periods of hyperoxia in the developing brain.\textsuperscript{87,88} Furthermore, clinical disorders may have periods of hyperoxia followed by cerebral hypoperfusion and hypoxia that can lead to cerebral injury with associated oxidative stress.\textsuperscript{89} In these circumstances, EPO also may be protective since it can promote neurite outgrowth\textsuperscript{90} and also may regulate hemoglobin levels that have recently been associated with cognitive decline.\textsuperscript{91} In other work, elevated EPO concentrations during infant maturation have been correlated with increased Mental Development Index scores\textsuperscript{92} and EPO may prevent toxic effects of agents used to control cognitive function such as haloperidol.\textsuperscript{93}

In addition, knowledge that EPO and its receptor are present in the neurovascular systems has generated great enthusiasm for the potential clinical applications of EPO for AD and related cardiac insufficiency\textsuperscript{94,99} and cardiac transplantation.\textsuperscript{96,97} In the nervous system, primary sites of EPO production and secretion are in the hippocampus, internal capsule, cortex, midbrain, cerebral endothelial cells (ECs) and astrocytes.\textsuperscript{73,74,98,99} Further work has revealed several other organs as secretory tissues for EPO that include peripheral ECs,\textsuperscript{100} myoblasts,\textsuperscript{101} insulinproducing cells\textsuperscript{102} and cardiac tissue.\textsuperscript{74,75} The EPOR also is expressed in primary cerebral ECs\textsuperscript{83,103} as well as in human umbilical veins, bovine adrenal capillaries and rat brain capillaries.\textsuperscript{100,104}

Despite the fact that EPO is a critical modulator of erythropoiesis, the presence of a diminished oxygen tension is required rather than a low concentration of red blood cells.\textsuperscript{7,78,79,105} Gene transcription of EPO is mediated by the transcription enhancer located in the 3'-flanking region of the EPO gene that specifically binds to hypoxia-inducible factor 1 (HIF-1).\textsuperscript{73,74} Yet, hypoxia is not the only condition that can alter the expression of EPO and the EPOR. A variety of cellular disturbances may lead to either increased or decreased EPO expression through the control of HIF, such as hypoglycemia, cadmium exposure, raised intracellular calcium, or intense neuronal depolarizations generated by mitochondrial ROS.\textsuperscript{99,106,107} Anemic stress, insulin release and several cytokines, including insulin-like growth factor, tumor
necrosis factor-\(\alpha\) (TNF\(\alpha\)),\(^{108}\) interleukin-1\(\beta\) (IL-1\(\beta\)) and interleukin-6 (IL-6)\(^{109}\) also can lead to increased expression of EPO and the EPOR\(^{73,74}\) and may provide a feed-back loop that is regulated by EPO such as TNF\(\alpha\).\(^{110}\)

**FoxO Transcription Factors**

**FoxO proteins and their regulation.** Mammalian forkhead transcription factors of the O class (FoxOs) function to either block or activate target gene expression.\(^{111}\) At least 100 forkhead genes and 19 human subgroups that range from FOXA to FOXS are now known to exist since the initial discovery of the fly Drosophila melanogaster gene forkhead.\(^{112}\) The original nomenclature for these proteins, such as forkhead in rhabdomyosarcoma (FKHR), the Drosophila gene fork head (fkh) and Forkhead RELated ACtivator (FREAC)-1 and -2, has been replaced.\(^{113}\) The current nomenclature for human Fox proteins places all letters in uppercase, otherwise only the initial letter is listed as uppercase for the mouse, and for all other chordates the initial and subclass letters are in uppercase.\(^{114}\)

FoxO proteins also may function as biomarkers. The activation of FoxO transcription factors during tumor invasion may suggest the initiation of cell pathways that are attempting to restrict neoplastic growth and represent a positive prognostic factor.\(^{111,115,116}\) However, reliance on any single biomarker may be imperfect and lead to initially unpredicted outcomes\(^{78,79,117}\) or the onset of detrimental apoptotic programs with forkhead transcription factors.\(^{116}\) A number of other pathways that occur in combination with a particular biomarker during oxidative stress also may also influence outcome. In the case of breast cancer, studies suggests that the release of androgens, cytokines or even changes in body mass and exercise can influence outcome as well as alter the predictability of a specific biomarker.\(^{118,119}\)

FoxO proteins (FoxO1, FoxO3, FoxO4 and FoxO6) are present throughout the body and are expressed in tissues of the reproductive system of males and females, skeletal muscle, the cardiovascular system, lung, liver, pancreas, spleen, thymus and the nervous system.\(^{105,111,115,116,128-127}\) Post-translational control of FoxO proteins employs pathways associated with ubiquitylation and acetylation.\(^{128,129}\) IKB kinase (IKK) can phosphorylate and block the activity of FoxO proteins, such as FoxO3a.\(^{113,115}\) This leads to the proteolysis of FoxO3a via the Ubdependent proteasome pathway.\(^{113,115,126,130,133}\) FoxOs also are acetylated by histone acetyltransferases that include p300, the CREB-binding protein (CBP), and the CBP-associated factor. FoxO proteins are deacetylated by histone deacetylases.\(^{115}\)

In addition to acetylation, and ubiquitylation, post-translational modulation of FoxO proteins also involves pathways

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**Figure 1.** Erythropoietin (EPO) regulates the intracellular trafficking of the forkhead transcription factor FoxO3a in endothelial cells (ECs) during oxygen glucose deprivation (OGD). EPO (10 ng/ml) was administered to ECs 1 hour prior to exposure of OGD for an 8 hour period. Immunofluorescent staining for FoxO3a at 6 hours following OGD was performed with primary rabbit anti-FoxO3a antibody followed by Texas red conjugated antirabbit secondary antibody. Nuclei of ECs were counterstained with DAPI. Control cells were untreated and not exposed to OGD. In control cells, FoxO3a remains primarily in the cytoplasm of cells with the nuclei visible in merged images and indicated by the white arrows. In contrast, OGD activates FoxO3a to translocate to the nucleus demonstrating FoxO3a in the cytoplasm and nuclei of these cells in merged images. However, EPO prevents nuclear translocation of FoxO3a by retaining FoxO3a in the cytoplasm similar to control cells with nuclei visible in merged images and indicated by the white arrows.
associated with phosphorylation. Protein phosphorylation is a critical pathway in the scheme for protein regulation. Akt is a primary mediator of phosphorylation of FoxO1, FoxO3a, and FoxO4 that can block activity of these proteins. Akt phosphorylation of FoxO proteins not only retains these transcription factors in the cytoplasm, but also leads to ubiquitination and degradation through the 26S proteasome. Interestingly, activation of Akt in pathways that involve EPO or FoxOs is usually cytoprotective, but may mediate other processes. For example, Akt either alone or through EPO can lead to cell proliferation, blood-brain barrier permeability, or cell protection during inflammation. Neurodegeneration, hyperglycemia, hypoxia, Aβ toxicity, excitotoxicity, cardiomyopathy, cellular aging and oxidative stress. In addition, Akt can prevent cellular apoptosis through the phosphorylation of FoxO proteins. Posttranslational phosphorylation of FoxOs, such as during EPO administration, will maintain FoxO transcription factors in the cytoplasm by association with 14-3-3 proteins and prevent the transcription of pro-apoptotic target genes (Fig. 1).

Modulation of Akt activity also controls apoptotic pathways of caspases that may offer an alternative mechanism to regulate FoxO proteins. Caspases are a family of cysteine proteases that are synthesized as inactive zymogens that are proteolytically cleaved into subunits at the onset of apoptosis. The activity of these proteins is tightly regulated. Cleavage of caspases 1 and 3 have been linked to the apoptotic pathways of genomic DNA cleavage, cellular membrane PS exposure and increased oxidized stress. Caspases 1 and 3 appear to offer a unique regulatory mechanism. For example, studies suggest that cell death pathways that rely upon FoxO3a also appear to involve caspase 3 activation. FoxO3a activity promotes caspase-induced apoptotic death, but inhibition of caspase 3 also can maintain the phosphorylated "inactive" state of FoxO3a to prevent cell injury. Other work has shown that caspase 3 activity and cleavage is promoted during transfection of a triple mutant FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of FoxO3a. Furthermore, FoxO3a may control early activation and subsequent apoptotic injury in microglia during Aβ exposure through caspase 3. Since Aβ exposure can facilitate the cellular trafficking of FoxO3a from the cytoplasm to the cell nucleus to potentially lead to "pro-apoptotic" programs by this transcription factor, one program in particular that may be vital for apoptotic injury appears to involve the activation of caspase 3. Aβ exposure leads to a rapid and significant increase in caspase 3 activity with 6 hours following Aβ administration, but that this induction of caspase 3 activity by Aβ requires FoxO3a, since loss of FoxO3a through gene silencing prevents the induction of caspase 3 activity by Aβ.

EPO, FoxOs, Nervous System Metabolism and Cognitive Impairment

Both EPO and FoxOs play a significant role during brain metabolism and metabolic disorders that can alter the progression of AD, such as during diabetes mellitus (DM). DM is a significant health concern for both young and older populations. Patients with DM can develop immune dysfunction, cognitive disorders, hepatic dysfunction, renal disease, hematological disease, neurodegenerative disorders and cardiovascular disease. Interestingly, the development of insulin resistance and the complications of DM can be the result of cellular oxidative stress. Furthermore, acute glucose swings in addition to chronic hyperglycemia can trigger oxidative stress mechanisms, illustrating the importance for therapeutic interventions during acute and sustained hyperglycemic episodes.

In regards to EPO during metabolic disorders, EPO administration has been shown both in diabetics as well as non-diabetics with severe, resistant congestive heart failure to decrease fatigue, increase left ventricular ejection fraction, and significantly decrease the number of hospitalization days. In vitro studies with vascular cells exposed to elevated glucose also have demonstrated that EPO can significantly improve EC survival in a 1.0 ng/ml range. EPO administration in patients also can significantly increase plasma levels of EPO well above this range of 1.0 ng/ml that has been associated with potential EPO cellular protection in patients with cardiac or renal disease, suggesting that the effects of EPO observed during in vitro studies may parallel the cellular processes altered by EPO in patients with metabolic disorders. Furthermore, EPO during elevated glucose and similar to other models of oxidative stress can block neuronal degeneration and apoptotic DNA degradation in ECs in vascular cell models. Protection by EPO is also related to the maintenance of mitochondrial membrane potential (ΔΨm). Loss of ΔΨm through the opening of the mitochondrial permeability transition pore represents a significant determinant for cell injury and the subsequent induction of apoptosis. EPO has the capacity to prevent the depolarization of the mitochondrial membrane that also affects the release of cytochrome c. Additional work suggests that proteins derived from the Drosophila Wingless (Wg) and the mouse Int-1 genes may be associated with cellular metabolic complications. The Wnt proteins are secreted cysteine-rich glycosylated proteins that can control cell proliferation, differentiation, survival and tumorigenesis. These genes are present in several cellular populations, such as neurons, cardiomyocytes, endothelial cells, cancer cells and preadipocytes. Abnormalities in the Wnt pathway, such as with transcription factor 7-like 2 gene, may impart increased risk for type 2 diabetes in some populations as well as have increased association with obesity. Yet, intact Wnt family members may offer glucose tolerance and increased insulin sensitivity as well as protect glomerular mesangial cells from elevated glucose induced apoptosis. These observations suggest a potential protective cellular mechanism for EPO through Wnt signaling. Cell culture studies demonstrate that the Wnt1 protein is necessary and sufficient to impart cellular protection.
during elevated glucose exposure. 165 EPO maintains the expression of Wnt1 during elevated glucose exposure and prevents loss of Wnt1 expression that would occur in the absence of EPO during elevated glucose. In addition, blockade of Wnt1 with a Wnt1 antibody can neutralize the protective capacity of EPO, illustrating that Wnt1 is a critical component in the cytoprotection of EPO during elevated glucose exposure. 165

In regards to FoxO proteins, analysis of the genetic variance in FOXO1a and FOXO3a on metabolic profiles, age-related diseases, fertility, fecundity and mortality in patients have observed higher HbA1c levels and increased mortality risk associated with specific haplotypes of FOXO1a. 179These clinical observations may coincide with the demonstration in human endothelial progenitor cells that elevated glucose levels can reduce post-translational phosphorylation of FOXO1, FOXO3a and FOXO4 and allow for the nuclear translocation of these proteins to initiate an apoptotic program in endothelial progenitor cells. 180 In experimental models, FoxO proteins may prevent the toxic effects of high serum glucose levels. 153, 115 Interferon-gamma driven expression of tryptophan catabolism by cytotoxic T lymphocyte antigen 4 may activate Fox3a to protect dendritic cells from injury in nonobese diabetic mice. 185 Additional studies have demonstrated that adipose tissue-specific expression of Foxo1 in mice improved glucose tolerance and sensitivity to insulin during an elevated fat diet. 182 FoxO proteins also may protect against diminished mitochondrial energy levels known to occur during insulin resistance such as in the elderly populations. 153, 134, 160 In caloric restricted mice that have decreased energy reserves, Foxo1, Foxo3a and Foxo4 mRNA levels were noted to progressively increase over a two year course. 122 These observations complement studies in Drosophila and mammalian cells that demonstrate an increase in insulin signaling to regulate cellular metabolism during the upregulation of FoxO1 expression. 183

It should be noted that the ability for FoxO proteins to maintain proper physiologic controls over cellular metabolism might be limited and occur only during specific circumstances. For example, mice with a constitutively active Foxo1 transgene have increased microsomal triglyceride transfer protein and elevated plasma triglyceride levels. 184 Studies in cardiomyocytes also suggest detrimental results with enhanced FoxO activity. Increased transcriptional activity of FoxO1, such as by the Sirt1 activator resveratrol, can diminish insulin mediated glucose uptake and result in insulin resistance. 185 Overexpression of Foxo1 in skeletal muscles of mice also can lead to reduced skeletal muscle mass and poor glycemic control, 186 illustrating that activation of FoxO proteins also may impair cellular energy reserves. Other studies that block the expression of Foxo1 in normal and cachectic mice 187 or reduce Foxo3 expression 188 show the reverse with an increase in skeletal muscle mass or resistance to muscle atrophy. With this in mind, one potential agent to consider for the maintenance of cellular metabolism in patients is nicotinamide. 13, 38 an agent that also can inhibit FoxO protein activity. 150 In patients with DM, oral nicotinamide protects β-cell function, prevents clinical disease in islet-cell antibody-positive first-degree relatives of type-1 DM, and can reduce HbA1c levels. 13, 38, 153 Nicotinamide, which is closely linked to cell longevity pathways, 189, 190 may derive its protective capacity through two separate mechanisms of post-translational modification of FoxO3a. Nicotinamide not only can maintain phosphorylation of FoxO3a and inhibit its activity, but also preserve FoxO3a integrity to block FoxO3a proteolysis that can yield pro-apoptotic amino-terminal fragments. 190

### EPO, FoxO and Neurovascular Survival

EPO and FoxO proteins can directly govern cell survival that can affect the progression of AD and cognitive loss. With EPO, it can prevent cell injury during AD and Aβ cell injury, hypoxia, 45, 143, 191, 192 excitotoxicity, 197, 199 parasitic disease, 200, 202 endotoxin shock, 203, 204 free radical exposure, 7, 63, 198 cardiac disease, 205, 206 amyloid toxicity, 143, 192 and pulmonary disease. 207, 208 EPO also represents a potential option for the prevention of retinal degeneration or neovascularization 209-212 as well as glaucoma. 213 In the CNS, systemic application of EPO also can improve functional outcome and reduce cell loss during spinal cord injury, 214, 215 traumatic cerebral edema, 216 cortical trauma 217 and epileptic activity. 82, 218, 219

EPO also can reduce cytokine gene expression in endothelial cells exposed to tumor necrosis factor, 165 prevent ulcer progression in cases of scleroderma, 220 reduce inflammation in murine arthritis models, 223 and block primary microglial activation and proliferation 24, 26, 35 during oxidative stress 40, 143 to prevent phagocytosis of injured cells through pathways that involve cellular membrane PS exposure, protein kinase B, 24 and the regulation of caspases. 40, 63, 222 EPO can directly inhibit several pro-inflammatory cytokines, such as IL-6, TNFα and monocyte chemoattractant protein 1, 74, 223 and reduce leukocyte inflammation. 224 EPO also may foster the preservation of microglial cells for neuronal and vascular restructuring by preventing apoptotic injury in microglia, 34, 225

In contrast to EPO cytoprotection, FoxO transcription factors usually lead to apoptosis during oxidative stress. 5 For example, forkhead transcription factors such as FoxO1 and FoxO3a must be present for oxidative stress to result in apoptotic cell injury. 226 FoxO3a in conjunction with JNK also has been shown to modulate an apoptotic ligand activating a Fas mediated death pathway in cultured motoneurons, 227 to lead to apoptosis through tumornecrosis-factor-related apoptosis-inducing ligand (TRAIL) and BH3-only proteins Noxa and Bim in neuroblastoma cells, 51 and to promote pro-apoptotic activity of p53. 228 In addition, loss of FoxO expression during oxidative stress is protective to cells. Protein inhibition or gene knockdown of FoxO1 or FoxO3a can lead to reduction in ischemic infarct size in the brain, 229 mediate protection of metabotropic glutamate receptors during vascular injury, 149 enhance pancreatic β-cell or neuronal survival through NAD+ precursors during oxidative stress, 190 and provide trophic factor protection with EPO 31 and neurotrophins. 230

Furthermore, similar to pathways tied to EPO and Wnt, the canonical Wnt pathway 231, 232 that involves β-catenin 99, 171 also appears to link FoxO proteins and Wnt signaling together. 30 For example, in relation to AD, 233 Aβ is toxic to cells 43, 143, 234 and is associated with the phosphorylation of FoxO1 and FoxO3a that can be blocked with ROS scavengers. 35 A common denominator
in the pathways linked to Aβ toxicity involves Wnt signaling and β-catenin. β-catenin may increase FoxO transcriptional activity and competitively limit β-catenin interaction with members of the lymphoid enhancer factor/T cell factor family. This may lead to cell injury, since β-catenin has been demonstrated to be necessary for protection against Aβ toxicity in neuronal cells. However, not all conditions with FoxOs may lead to cell demise. Some studies suggest that the loss of FoxO1, FoxO3a and FoxO4 protein expression may actually lead to an increase in free radical release that can be responsible for oxidative stress. In addition, FoxO proteins also may influence early apoptotic membrane PS externalization. The ability to regulate early apoptotic membrane PS exposure and inflammatory cell activity can ultimately affect cell survival since activated immune cells can lead to the phagocytic removal of injured cells. Furthermore, FoxO proteins may be protective during aging and exercise, since FoxO3a activity may enhance vascular smooth muscle antioxidant properties in aged animals and be beneficial to the cardiovascular system during physical exertion.

**Future Perspectives**

As biomarkers for disease onset and progression as well as candidates for the treatment of numerous disorders, EPO and FoxO transcription factors generate excitement for the potential to yield new strategies for the treatment of neurovascular injury and cognitive disorders. Yet, some considerations for EPO exist. In addition to the problems associated with EPO abuse and gene doping, EPO has been correlated with the alteration of red cell membrane properties leading to a cognitive decrement in rodent animal models. Development of potentially detrimental side-effects during EPO therapy, such as for cerebral ischemia with increased metabolic rate and blood viscosity, could also severely limit the use of EPO for neurovascular diseases. As a result, alternate strategies have been suggested. New proposals examine the role of targeted bioavailability for EPO such as in bone marrow stromal cells genetically engineered to secrete EPO and controlled release of EPO from encapsulated cells. The passage of EPO entry into the CNS continues to attract significant interest as well as does the use of novel intranasal routes for EPO administration. The development of therapies for EPO to reduce erythropoietic activity and the potential associated vascular complications have also been put forth as new directions for treatment. Yet, these lines of investigation are not without limitations, since chemical derivatives of EPO can become absent of clinical efficacy as well as possibly loosen the ability to promote sustainable cytoprotective effects, such as neurogenesis and angiogenesis.

Other work also offers additional support for the use of FoxO proteins as biomarkers of neurovascular injury that can occur during AD and cognitive loss. Down regulation of the phosphatidylinositol 3 kinase and Akt pathways have been associated with increased transcript levels for FOXO1a and FOXO3a in cell loss scenarios. The known mutations in FoxO proteins that exist in several disease entities may provide novel insights for the new treatment strategies. Future analysis in larger populations of patients with metabolic disease and cognitive loss could strengthen our understanding of the role of FoxO proteins in these disorders. In addition, targeting the activity of FoxO1, FoxO3a or FoxO4 in vascular cells may prevent the onset of pathological neointimal hyperplasia that may result in atherosclerosis and cognitive loss. Recent studies also suggest that the utilization and combination of multiple biomarkers may improve risk assessment for patients suffering from a number of disorders. These studies illustrate that FoxO proteins may serve as biomarkers of disease activity such as in individuals with imminent cardiac failure.

As combined therapeutic entities and biomarkers, EPO and FoxO proteins share a number of pathways to offer novel therapeutic strategies for a broad range of disorders. Future studies that involve basic research as well as clinical trials are warranted for EPO and FoxO proteins. Yet, critical to this process is the clear focus upon the intricate cellular pathways governed by EPO and FoxOs to uncover the benefits and risks of these agents for development of proper therapies to prevent the onset or progression of AD and cognitive loss.

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