

Review Article

Targeting Glycogen Synthase Kinase-3 β for Therapeutic Benefit against Oxidative Stress in Alzheimer's Disease: Involvement of the Nrf2-ARE Pathway

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Specific regions of the Alzheimer's disease (AD) brain are burdened with extracellular protein deposits, the accumulation of which is concomitant with a complex cascade of overlapping events. Many of these pathological processes produce oxidative stress. Under normal conditions, oxidative stress leads to the activation of defensive gene expression that promotes cell survival. At the forefront of defence is the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates a broad spectrum of protective genes. Glycogen synthase kinase-3 β (GSK-3 β) regulates Nrf2, thus making this kinase a potential target for therapeutic intervention aiming to boost the protective activation of Nrf2. This paper aims to review the neuroprotective role of Nrf2 in AD, with special emphasis on the role of GSK-3 β in the regulation of the Nrf2 pathway. We also examine the potential of inducing GSK-3 β by small-molecule activators, dithiocarbamates, which potentially exert their beneficial therapeutic effects via the activation of the Nrf2 pathway.

1. Alzheimer's Disease and Oxidative Stress

Alzheimer's disease is a common age-associated dementia characterized by pathological, progressive loss of neurons and synapses, accumulation of intra- and extracellular protein deposits, and gliosis. The amyloid hypothesis of AD postulates that amyloid-beta ($A\beta$) deposition and neurotoxicity play a causative role in AD [1]. Although the mechanisms through which $A\beta$ exerts its toxicity are numerous [2], it appears that oxidative injury is central in the pathogenesis of AD [3–5].

Oxidative stress results from an imbalance between the production and removal of physiologically important molecules collectively called reactive oxygen species (ROS) [6–8]. Oxidative stress damages all cellular macromolecules and when uncontrolled, leads to irreparable oxidative injury

and cell death. The imbalance between the production and removal of ROS occurs when endogenous defence systems are overwhelmed or exhausted, usually due to disease or as part of normal aging. It is thus not surprising that oxidative stress is involved in the pathogenesis of several neurodegenerative disorders, including AD.

Oxidative stress is a central feature of AD, and, in fact, it may even be one of the first pathogenic events during disease progression [5]. Markers of oxidative damage such as protein carbonyls [9, 10] and elevated lipid peroxidation [11, 12] precede pathological changes and are found in the brains of AD patients. Importantly, antioxidant defence is impaired in mouse models of AD; the levels and activities of protective enzymes including superoxide dismutase (SOD) and glutathione peroxidase are altered [13].

2. GSK-3 β and AD

GSK-3 β is a serine/threonine kinase that regulates diverse cellular functions ranging from glycogen metabolism to gene transcription and cell survival [14, 15].

Several lines of evidence directly link GSK-3 β to the neuropathology of AD [14, 16, 17]. In fact, the recently described GSK hypothesis of AD depicts how overactivation of GSK-3 β accounts for the pathology of AD [16]. GSK-3 β expression is elevated in the brain of AD patients and is implicated with at least three major pathological hallmarks of the disease [18, 19]. First, overactivity of GSK-3 β accounts, at least in part, for tau hyperphosphorylation [20]. In human brain, active GSK-3 β has been detected in neurons loaded with neurofibrillary tangles [21], and *in vitro* evidence link increased GSK-3 β activation to tau hyperphosphorylation. In fact, A β can activate GSK-3 β , leading eventually to increased tau phosphorylation and loss of microtubule binding. In agreement with the *in vitro* evidence, mice overexpressing GSK-3 β display prominent tau hyperphosphorylation [22]. Secondly, acetylcholine synthesis is suppressed by GSK-3 β , thus implicating the kinase in the cholinergic deficit characteristic of the disease [23]. This effect has been shown to be mediated by GSK-3 β -dependent inactivation of pyruvate dehydrogenase (PDH), leading to the depletion of acetyl coenzyme A, an important precursor in acetylcholine synthesis [23]. Thirdly, overactivity of the kinase causes increased production of toxic A β . GSK-3 β has been shown to interact with presenilin, thus increasing the production of the amyloid precursor protein (APP) and subsequently toxic A β . The increased production of A β then leads to synaptic deficits and memory impairment [24–27]. GSK-3 β can also directly phosphorylate APP *in vitro* [28], and, interestingly, AD-related mutated presenilin-1 is able to both directly and indirectly activate GSK-3 β [29, 30]. Not surprisingly, the inhibition of GSK-3 β blocks the accumulation of A β and reduces plaque burden in transgenic mice modelling AD [24], while the overexpression of GSK-3 β causes memory deficits [31, 32]. Certain antibodies that reduce brain A β burden also mediate their protective effect by inhibiting GSK-3 β activation [33]. Finally, GSK-3 β is an important mediator of apoptosis [34], thus its dysregulation may also directly contribute to AD-associated neuronal loss.

GSK-3 β phosphorylates a diverse group of substrates, including over 20 transcription factors [14, 35]. It is hypothesized that GSK-3 β -mediated impaired activation of transcription factors compromises the ability of cells to respond adequately to stressful conditions. Indeed, GSK-3 β is inhibitory towards the activation of transcription factors such as heat shock factor-1 [36, 37] and cyclic AMP response element-binding protein [14, 38], which are important in cell survival mechanisms after potentially toxic insults [39–41]. However, it is important to note that GSK-3 β activation may also affect cell survival by other mechanisms. For example, activated GSK-3 β can inactivate an important mediator in the citric acid cycle, PDH, and may thus impair the energy supply in neurons [23].

As the majority of the transcription factors controlled by GSK-3 β are involved in cellular survival pathways, the

modulation of transcription factors by GSK-3 β is likely an important survival mechanism against various stresses, including oxidative stress. It is interesting to note that oxidative stress itself may also be related to GSK-3 β activation. For example, oxidative stress induces overactivation of GSK-3 β in neuronal cells [42], while the inhibition of GSK-3 β is involved in the control of oxidative stress in neuronal hippocampal cell lines [43].

3. Endogenous Defence against Oxidative Stress

Under normal conditions, oxidative stress leads to the activation of a battery of defensive gene expression that leads to detoxification, prevention of free radical generation and cell survival [44]. At the forefront of defence against oxidative stress is the transcription factor named nuclear factor erythroid 2-related factor (Nrf2). Human Nrf2 was first isolated in 1994 from a hemin-induced K562 erythroid cell line and showed high sequence specificity to the known p45 subunit of NF-E2 [45, 46]. Nrf2 regulates a broad spectrum of enzymes and proteins involved in the disposition of harmful compounds causing oxidative stress. The classes of genes regulated by Nrf2 affect such diverse functions as detoxification of electrophiles, free radical metabolism, glutathione metabolism, proteasome function, and calcium homeostasis [47]. Microarray analyses have revealed numerous Nrf2-dependent genes that confer protection against oxidative stress *in vitro* [48, 49, 49, 50]. Some of the well-characterized cytoprotective genes controlled by Nrf2 include heme oxygenases (HOs), NAD(P)H:quinone oxidoreductases, superoxide dismutases (SODs) and the rate-limiting enzymes of glutathione synthesis consisting of catalytic (GCLC) and modifier (GCLM) subunits [51–53].

Transcription factors such as Nrf2 are considered key targets of numerous signalling pathways because they have the critical role of transferring information from the extracellular environment to the nucleus to regulate multiple functions. The induction of the Nrf2-controlled protective response requires at least three essential components; antioxidant response elements (AREs), Nrf2, and Kelch ECH-associating protein 1 (Keap1) [47]. AREs are enhancer sequences that control the basal and inducible expression of protective genes in response to oxidative stress, xenobiotics, heavy metals and ultraviolet light [44, 54, 55]. Nrf2 is the principal transcription factor that binds to the ARE and induces the expression of ARE-driven cytoprotective genes. Keap1 is a cytosolic, actin-associated protein that suppresses the activity of Nrf2 by sequestering it in the cytoplasm and by targeting it for proteasomal degradation.

The abundance of Nrf2 inside the nucleus is constantly regulated by positive and negative stimuli that affect nuclear import and export, binding to the ARE, as well as the degradation of Nrf2 [44]. It is well known that Keap1 [56] is an inhibitor of Nrf2 that targets Nrf2 for degradation and thus promotes low, basal expression of cytoprotective genes under normal physiological conditions. Previously, it was thought that oxidative stimuli activate Nrf2 by promoting its dissociation from Keap1; however, as the binding affinity between the two proteins is not affected by oxidative stress

[57–59], it is now understood that the main function of Keap1 is to serve as an adapter for the Cullin3/Ring Box 1 E3 ubiquitin ligase complex [60, 61]. Keap1 binding to Cullin3 and Nrf2 leads to the ubiquitination and degradation of Nrf2 through the 26S proteasome [62, 63]. Keap1 also functions as ROS sensor molecule, the cysteine residues of which are modified upon conditions of oxidative stress [64, 65]. Oxidative stress impairs the ability of Keap1 to target Nrf2 for degradation, most likely by triggering an alteration in Keap1 conformation.

4. Nrf2 in AD

The activation of the Nrf2-ARE pathway is beneficial in animal models of various diseases of the central nervous system, including chronic neurodegenerative diseases such as Parkinson's disease and acute insults such as brain ischemia and brain trauma [66–69]. However, in comparison to other neurodegenerative disorders, the role of Nrf2 in AD has received relatively little attention. Currently, it is known that while Nrf2 is not a susceptibility gene for AD, common variants of the gene encoding Nrf2 may affect disease progression [70]. In the human AD brain, the amount of nuclear Nrf2 is reduced in the hippocampus [71]. Histochemical analyses demonstrate that Nrf2 predominantly localizes to the cytoplasm of AD-affected hippocampal neurons, suggesting that despite oxidative stress, Nrf2-mediated transcription is not induced in AD patients. However, this study utilized brain tissue demonstrating full-blown AD pathology; it is not clear whether the finding is a cause or consequence of the ongoing pathological events and cell death. In fact, it has been suggested that the induction of Nrf2, and levels and activity of its cytoprotective target enzymes may display a time-dependent alteration in AD [66].

We recently showed that attenuation of the Nrf2-ARE pathway coincides with disease progression in the APDe9 transgenic mice modelling AD [72]. The Nrf2-pathway was impaired in transgenic AD mice concomitantly with increased brain A β burden. This AD-associated reduction in Nrf2 was recently confirmed by Choudry et al., who described a 50% reduction in Nrf2 levels in transgenic AD mice [73].

A growing body of literature suggests that Nrf2 is neuroprotective in AD. Induction of the Nrf2-ARE pathway by small therapeutic molecules protects against neuronal dysfunction and toxicity mediated by A β *in vitro* [72, 74–76]. Interestingly, Nrf2 can also exert its protective effects by suppressing oxidative stress-induced A β formation [74, 77] and by inducing the 26S proteasome, thus facilitating the removal of toxic A β [78]. Protection against A β toxicity by coffee extract has also been shown to occur via the induction of the Nrf2-ARE pathway in *Caenorhabditis elegans* [79].

In addition to assessing the effect of small molecule inducers of the Nrf2-ARE pathway on A β toxicity, we recently studied therapeutic and disease-modifying properties of Nrf2-ARE induction by gene transfer in transgenic mice modelling AD [80]. The long-term effect of Nrf2-ARE activation was studied *in vivo* by employing a gene therapy approach where human Nrf2 was directly injected into the

hippocampi of transgenic AD mice, an area of the brain important for learning and memory that is directly affected by AD pathology. Evident improvement in cognitive abilities was achieved when transgenic AD mice were treated with the Nrf2 vector at the age of 9 months and assessed in the Morris water maze 6 months later. This effect was associated with the induction of the Nrf2-pathway, suggesting that strategies aimed at boosting the Nrf2-ARE pathway constitute a potential therapeutic approach for AD.

5. GSK-3 β in the Regulation of the Nrf2-ARE Pathway

As GSK-3 β controls a variety of targets in several cellular pathways, it is not surprising that the kinase is also implicated in the regulation of Nrf2. GSK-3 β exerts a negative form of regulation on Nrf2 by controlling its subcellular distribution [81] (Figure 1). Long-term exposure to hydrogen peroxide causes downregulation of Akt, activation of GSK-3 β , and translocation of Nrf2 from the nucleus to the cytosol, thus limiting the antioxidant response of cells [82]. This is particularly important in conditions of prolonged oxidative stress, such as AD, and highlights the importance of this kinase in chronic neurodegenerative disorders. The inhibition of GSK-3 β results in nuclear accumulation and the elevation of transcriptional activity of Nrf2 [82], indicating that GSK-3 β is a fundamental element of Nrf2-ARE downregulation after oxidative injury. The mechanism of GSK-3 β -mediated Nrf2 inhibition appears to involve the tyrosine kinase Fyn, which is phosphorylated by activated GSK-3 β and leads to nuclear localization of Fyn. Activated Fyn phosphorylates tyrosine 568 of Nrf2 [83] in the nucleus, leading to Nrf2 export and dampening of protective gene transcription [83, 84]. Once excluded from the nucleus, Nrf2 is degraded. Very recently, it was shown that transfection with a constitutively active genetic variant of GSK-3 β completely inhibits nuclear accumulation of Nrf2, providing further support for the role of GSK-3 β in controlling Nrf2 activation [85].

Further evidence for the importance of GSK-3 β in the regulation of Nrf2 is demonstrated by the finding that activation of the muscarinic M1 receptor induces Nrf2 through a signalling cascade involving protein kinase C-mediated inhibition of GSK-3 β [86]. Moreover, GSK-3 β is known to regulate oxidative stress protein SKN-1, the functional counterpart of Nrf2, in *Caenorhabditis elegans* [87]. Taken together, these data suggest that increased activation of GSK-3 β leads to a dampening of the protective Nrf2-ARE pathway.

6. Dithiocarbamates as Nrf2-Inducing Pharmacological Agents Targeted to GSK-3 β Activation

Stimulation of the Nrf2-ARE pathway by small-molecule activators represents an appealing strategy to upregulate the endogenous defence mechanism of cells against oxidative stress. At least nine classes of Nrf2 inducers have been described [88] and several of these are protective in models of neurodegenerative diseases. However, more potent, safe,

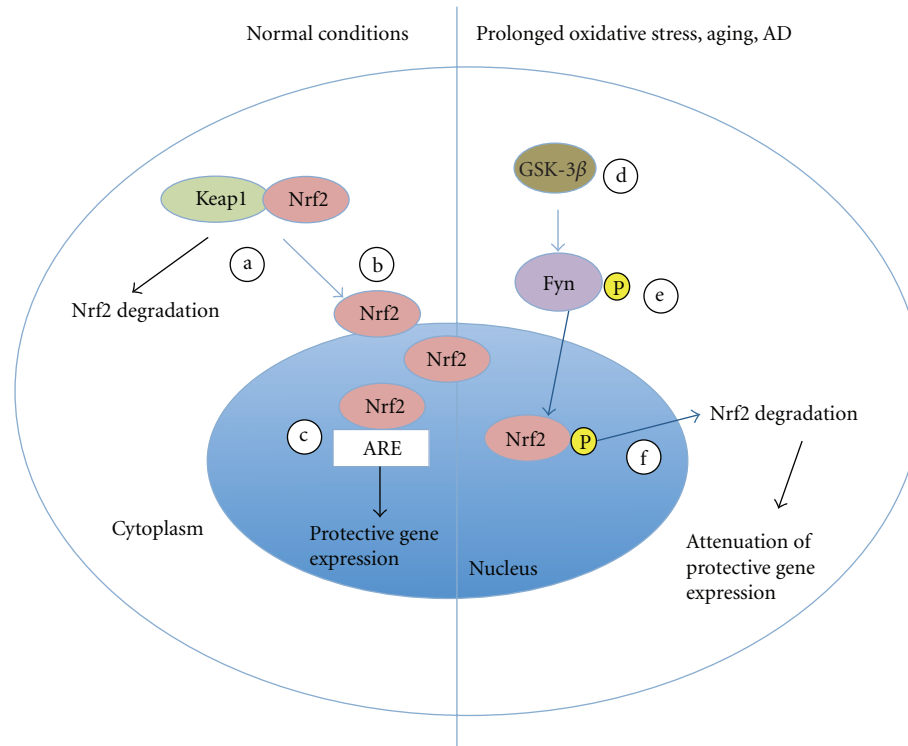


FIGURE 1: Induction of the Nrf2-ARE pathway under normal and pathological conditions: potential role of GSK-3 β as a negative regulator of Nrf2. (a) In normal conditions, actin-associated Keap1 sequesters Nrf2 in the cytosol and targets it for degradation. (b) Endogenously and exogenously generated ROS alter the interaction between Nrf2 and its repressor, resulting in the accumulation of Nrf2 in the cytoplasm. Nrf2 translocates into the nucleus. (c) An abundance of Nrf2 in the nucleus results in the binding of Nrf2 to the ARE and the increased expression of protective, Nrf2-controlled genes. (d) In conditions of prolonged oxidative stress, GSK-3 β is activated. (e) Activated GSK-3 β phosphorylates Fyn, causing nuclear translocation. (f) Nuclear Fyn phosphorylates Nrf2, leading to nuclear export of Nrf2 and degradation in the cytosol.

and specific activators of the Nrf2-ARE pathway that cross the blood brain barrier (BBB) need to be explored in relevant models of AD.

Dithiocarbamates are attractive drug candidates for many diseases as they are BBB permeant metal chelating compounds that possess antioxidant and anti-inflammatory properties [89] and are clinically approved for treatment of alcohol addiction (Antabus) and heavy metal poisoning. In addition to its well-characterized role in the inhibition of nuclear factor- κ B [90, 91], pyrrolidine dithiocarbamate (PDTC) has potent antioxidant properties and is able to scavenge ROS [90–93]. It is becoming increasingly evident that PDTC also has the potential to activate endogenous antioxidant gene expression. *In vitro* studies suggest that PDTC treatment results in the activation and nuclear translocation of Nrf2 [94, 95]. Moreover, the cytoprotective, Nrf2-controlled proteins, HO-1, GCLM, and SOD, are potently induced in response to PDTC *in vitro* [94, 96–98]. Interestingly, it has also been shown that PDTC can act as a pro-oxidant [99]. Indeed, PDTC induces apoptosis in several *in vitro* models [100, 101, 101, 102] and may also be toxic to neurons *in vivo* [103]. Whether the action of PDTC is anti- or pro-oxidant has been reported to depend on the dose of PDTC and the presence of metal ions [104]. It may well

be that PDTC can exert its effects on Nrf2-mediated gene transcription by mimicking an oxidative insult (and thus acting as a pro-oxidant) that triggers Nrf2 activation.

PDTC is known to reduce activated GSK-3 β signalling in neonatal hypoxia-ischemia [105]. We also showed that while the activity of GSK-3 β is increased in the brains of transgenic AD mice [106], a 7-month treatment with PDTC reduces the amount of active GSK-3 β with concomitant improvement in the spatial learning of the treated mice. This effect may involve the metal-chelating ability of PDTC. It is known that PDTC can transport extracellular copper into cells [107] and depending on the situation at hand, this may have different effects. For example, copper transport into cells is most likely beneficial in AD animal models [108–110], where pools of intracellular copper are depleted or unevenly or inefficiently distributed within the brain parenchyma. In contrast, under normal conditions, an increase in cellular copper levels may cause an increase in free radical production and apoptosis [111, 112]. As copper can induce the Akt pathway, we hypothesize that the PDTC-mediated increase in intracellular copper could trigger the phosphorylation of Akt, leading to reduced GSK-3 β activity.

To analyze the association of Nrf2 with the beneficial effect of PDTC treatment, we assessed the potential of

PDTC in protection against $A\beta$ toxicity in primary cultures prepared from Nrf2 knockout mice. While PDTC protected against $A\beta$ toxicity in wild-type neuronal cultures, the beneficial effect was abolished in cultures prepared from knock-out mice (Kanninen, K et al., unpublished data). Moreover, PDTC treatment of neuronal cultures induced Nrf2 target genes (Kanninen, K et al., unpublished data). Taken together, these data indicate that Nrf2 is required for the beneficial effect of PDTC against $A\beta$ toxicity *in vitro* and suggest that Nrf2-ARE induction may be associated with the beneficial effect of PDTC in AD. However, further studies are required to clarify and specify the involvement of GSK-3 β in aberrant regulation of Nrf2 in AD.

7. Targeting GSK-3 β for Therapeutic Benefit in AD: Involvement of the Nrf2-ARE Pathway

While the detailed mechanisms behind the impairment of the Nrf2-ARE pathway in transgenic AD mice remain unresolved, it is possible that it involves GSK-3 β . Considering that GSK-3 β can inactivate Nrf2 [81, 86], it is conceivable that the Nrf2-ARE pathway is dampened in the aged AD transgenic mouse brain through the increased activity of GSK-3 β . In fact, long-term oxidative stress causes GSK-3 β activation and reduces nuclear Nrf2, suggestive of downregulation of the Nrf2-ARE pathway [82]. This hypothesis is further supported by the finding that pharmacological treatments, which inhibit GSK-3 β , have been reported to reduce $A\beta$ pathology and cognitive impairment in AD mice [24]. Moreover, lithium, a GSK-3 β inhibitor, has been shown to promote the transcriptional activity of Nrf2 [82].

While modulating the activity of GSK-3 β is known to be beneficial in models of AD, studying the mechanism of action of modulators of the kinase is important in understanding the diverse pathways GSK-3 β is involved in and the numerous effects modulation may have. For example, treatment with small molecules such as PDTC prevents cognitive impairment in transgenic AD mice [106], not only by the inhibition of GSK-3 β , but also potentially via the activation of the Nrf2-ARE pathway.

8. Concluding Remarks

Due to the major social and economical burden caused by the aging of populations and the subsequent increase in the incidences of neurodegenerative diseases, potential novel targets for effective AD therapeutics are urgently needed. Despite extensive research and knowledge that oxidative stress is a central pathological feature of AD, several therapeutic approaches targeted to this aspect of disease have failed, most likely because they have targeted only one aspect such as the decline of a single antioxidant. Considering the complexity of the antioxidant system, it seems reasonable to consider that the induction of endogenous protective pathways, such as the Nrf2-ARE pathway against oxidative stress, is a viable strategy for delaying the progression of injury and cell death.

It is clear that understanding the mechanisms of regulation of the Nrf2-ARE pathway and how these mechanisms

are impaired in disease is central in deciphering how we can modulate this protective pathway against oxidative stress associated with AD. While several other regulatory pathways of Nrf2-ARE have been described and GSK-3 β modulation in AD is also known to be beneficial in several ways, studying the influence of GSK-3 β on Nrf2-ARE is certainly an important path to pursue in order to better understand how to combat the oxidative stress associated with AD.

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