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ASK1 and Its Role in Neurodegenerative Diseases

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1. Introduction

The apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed serine/threonine protein kinase and one of more than 20 members that make up the triple MAP kinase (MAP3K) family of enzymes. Over the past decade, genetic studies have revealed that ASK1 plays a pivitol role in the cellular response to a wide variety of environmental and biological stressors including; reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), endoplasmic reticulum (ER) stress caused by protein aggregation, influx of calcium ions, and receptor-mediated signals transduced via lipopolysaccharides (LPS), Fas ligand, cytokines (TNFα) and certain G protein-coupled receptor (GPCR) agonists [1-5]. In addition, exogenous expression of ASK1 in cells has shown that ASK1 signaling engages the intrinsic apoptosis pathway promoting cytochrome *c* release from mitochodria and subsequent activation of caspase 3 and 9 [1, 6, 7]. Conversely, ASK1 deficient cells are resistant to cell death induced by oxidative and ER stress, indicating that ASK1 acts as the lynch pin in certain forms of stress-induced cell death [8].

Once activated, ASK1 relays cellular stress signals via the classical three tierd mitogen activated protein kinase (MAPK) signaling cascade, whereby a MAP3K phosphorylates and activates a MAP2K, that in turn phosphorylates and activates a MAPK [9] (Figure 1). More specifically, the ASK1 signaling axis activates the p38 and the c-jun NH2-terminal kinases (JNK) family of MAPKs, via activation of MKK3/MKK6 and MKK4/MKK7 respectively [1, 2, 4]. In addition to its role in the cellular stress response, ASK1 also regulates physiological processes including neuronal differentiation, synaptic plasticity and the innate immune response [10-13]. Thus, ASK1 acts as an important regulator of several important biological processess and not surprisingly, ASK1 activation is under tight regulatory control.

Regulation of ASK1 activity is accomplished via a number of mechanisms including; protein-protein interactions as well as both spatial and temporal control. Firstly, more than 30 ASK1 interacting partners have been shown to regulate ASK1 activity (either positively or negatively) by posttranslational modifications and/or by inducing conformational changes through protein-protein interactions. Secondly, ASK1 signaling complexes are located in both the cytoplasm and mitochondria [14], with nuclear translocation observed upon stress induction indicating that ASK1 localization might also dictate the biological outcome [15,16] and thirdly, duration of ASK1 signaling can influence the nature of the

cellular response. In this context, defects in the fine tuning of ASK1 activity can contribute to a number of pathological conditions including inflammatory, cardiac and several neurodegenerative diseases. In this chapter, we will discuss in detail the molecular mechanisms that regulate ASK1 activity, and focus on the contribution of ASK1 to neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, Polyglutamine (polyQ) Diseases, amyotrophic lateral sclerosis and stroke, together with the potential of ASK1 as a therapeutic target for the treatment of such disorders.

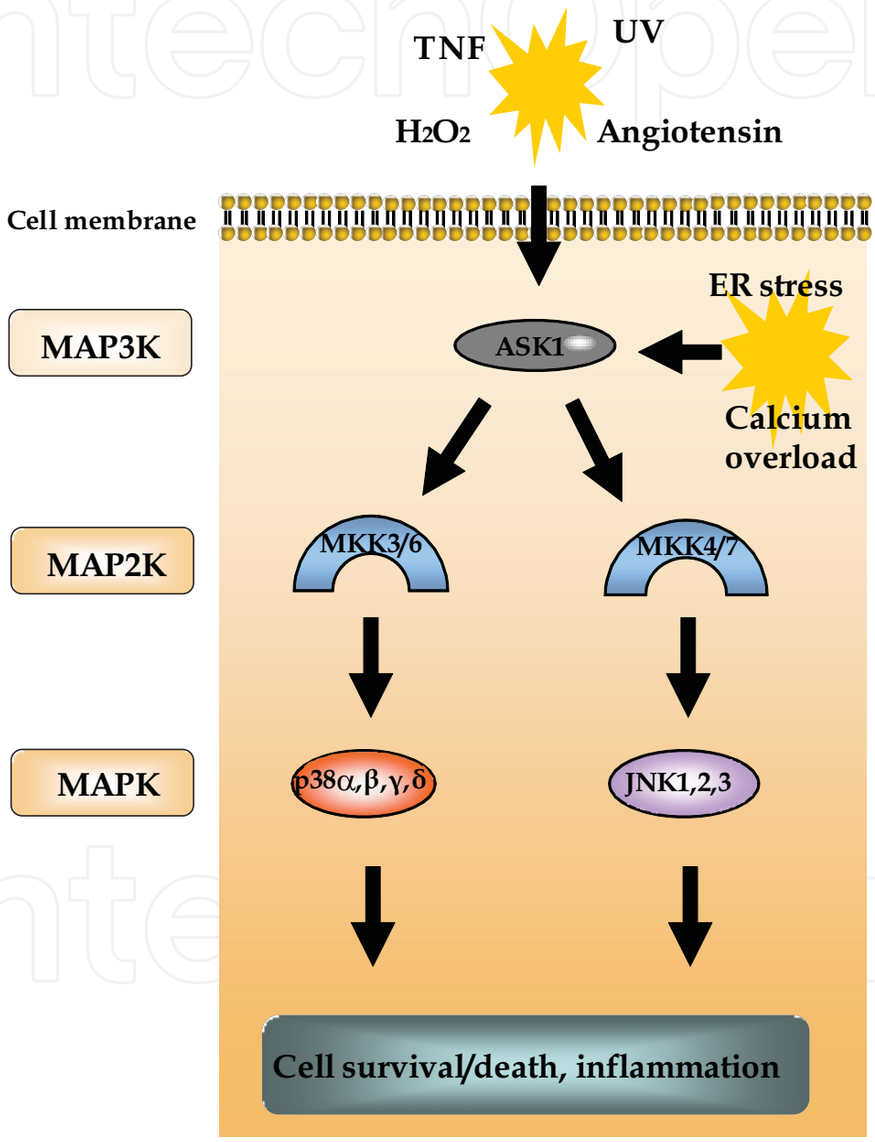

Fig. 1. ASK1, along with at least 20 other kinases belongs to the MAP3K family of enzymes. MAP3K selectively activate MAP2Ks with in turn activate members of the MAPK family. There are 7 MAP2K and 11 MAPK family members with the global MAPK family constituting 8% of the kinome. Specifically, ASK1 responds to multiple extracellular and intracellular stimuli by selectively phosphorylating MKK3/6 and/or MKK4/7 leading to the specific activation of the p38 and JNK MAPK pathways

2. Regulation of ASK1 activity

Based on sequence homology, three human ASK1 related genes have been identified, namely ASK1 (MAP3K5), ASK2 (MAP3K6) and ASK3 (MAP3K15). In this review we will focus specifically on the biology of ASK1, commenting only on ASK2 and 3 where appropriate. ASK1 is a 170 kDa protein composed of an inhibitiory N-terminal domain, an internal kinase domain and a C-terminal regulatory domain. Although the primary stuctures of the N- and C regulatory domains within the ASK family exhibit a fair degree of divergence, those within the kinase domains are highly conseved. High resolution crystal structure studies revealed that the ASK1 catalytic domain structure displays a typical protein kinase fold, comprised of five β sheets and helix αC constituting the small lobe and a larger mainly α helical C-terminal lobe. The hinge region connecting the two domains lines the catalytic ATP binding site and although catalytically active, the recombinant enzyme adopts a non-active conformation in the crystal state [17].

It is well established that both the subcellular localization as well as the magnitude and duration of MAPK activation are important for determining cellular fate. Thus, specific mechanisms may enable ASK1 modules to be rapidly activated or inactivated in a spatial and temporal manner. Increasing evidence indicates that depending on the stimulus and the cellular context, ASK1 activity is tightly regulated by multiple and distinct molecular events including phosphorylation/dephosphorylation of key residues, protein-protein interactions, and ubiquitination, resulting in ASK1 degradation and feedback regulation. Defects in these mechanisms may lead to aberrant ASK1 activity and to certain pathologies in humans.

3. Modulation of ASK1 activity by postranslational modification

In resting cells, ASK1 forms homo-oligomers through protein interactions via the C-terminal coiled-coiled domain (CCC). Cell-based and biochemical studies have demonstrated that ASK1 is associated with a number of interacting proteins which can lead to the formation of high molecular weight complexes (1000-3000 kDa), designated the ASK1 signalosome [18, 19]. To date, over 30 proteins have been shown to interact with ASK1 and regulate its activity. While the presice nature of the ASK1 signalosome remains to be fully characterised, it is postulated to be highly dynamic, serving as a foundation for the assembly of specific signaling modules and the subsets of regulatory proteins that it recruits depends upon the context of the initial stress.

Through genetic screening for ASK1-binding proteins, the redox protein Thioredoxin (Trx1) was one of the first ASK1 interacting proteins identified and has been shown to play a key role in the regulation of ASK1 in response to oxidative stress induced by H_2O_2 or tumor necrosis factor-α (TNFα) (Figure 2) [20, 21]. The thiol reductase activity of Trx1 is provided by cysteines 32 (Cys32) and 35 (Cys35) which forms a redox cataylitic CXPC motif [22]. Several studies demonstrated that tight association of a reduced form of Trx1 with the N-terminus of ASK1 suppresses ASK1 kinase activity by inhibiting N-terminal interactions between ASK1 monomers. The interaction has been shown to involve the catalytic cysteines Cys32 and Cys35 of Trx1, as mutation of either is sufficient to inhibit the dissociation of Trx1 from ASK1 [21]. Moreover, antioxidants such as catalase and N-acetylycysteine (NAC) block the release of Trx1 from ASK1 after challenge with H_2O_2, preventing ASK1 activation and associated cell death [23, 24]. These studies led to a model whereby upon H_2O_2 stimulation, Trx-1 is oxidized on Cys32 and Cys35 promoting formation of an intramolecular disulfide

bond beetween these two cysteines. This allows dissociation of Trx1 from ASK1 promoting multimerization and activation of ASK1 [20, 23].

Studies conducted by Nadeau *et al*., revealed an alternative mechanism for the function of Trx1 in the regulation of ASK1 in response to H_2O_2. These studies demonstrated that H_2O_2 induces ASK1 oxidation leading to the formation of interchain disulfide bond-linked ASK1 multimers [25, 26]. These authors demonstrated that changing all oxidation sensitive cysteine residues responsible for disulfide bond-linked multimers, prevented H_2O_2-induced ASK1 proapototic activity. Specifically, the cysteine residue at position 250 (Cys250) in ASK1 was identified as an essential residue for JNK activation in response to H_2O_2-induced stress. Recently, redox sensitive molecules such as the Parkinson's associated protein DJ-1 (a.k.a PARK7) and Peroxiredoxin-2 have also been shown to attenuate ASK1 activity in response to toxic stress in dopaminergic neurons [27-29]. Interestingly, mutation analyses demonstrated, that the ER stress-inducing agent thapsigargin, while inducing ASK1 activation (as determined by Thr838 phosphorylation) was observed to be independent of Cys250 [25]. In attempting to address the role of Cys250 and the role of Trx1 in regulating

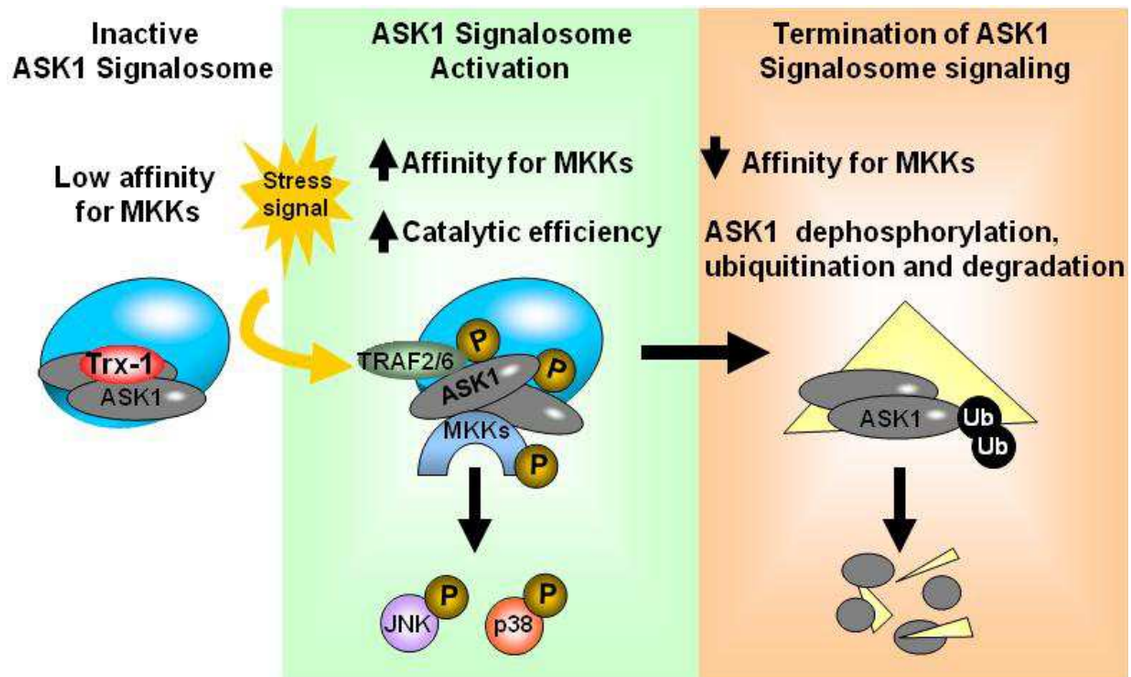

Fig. 2. ASK1-MKKs-JNK/p38 signaling cascade. Under basal conditions, ASK1 forms a high molecular mass complex with multiple interacting proteins including thioredoxin (Trx). In response to oxidative stress, ER stress, or calcium overload, firstly Trx is oxidized and released from the signalosome, secondly the TNF receptor associated factors 2 and 6 (TRAF2/6) are recruited to the complex leading to ASK1 autophosphorylation/transphosphorylation and activation. Subsequently, the affinity of the signalosome for its substrates, (MKKs) is increased, favoring ASK1-MKK6/4 interaction and MKK6/4 phosphoryation which in turn activate JNK/p38. Depending upon the cell type and initial stressor, a complex ASK1 regulatory mechanism is invoked, balancing the processes of phosphorylation/dephosphorylation together with ubiquitination/deubiquitination which fine tunes the specificity and duration of the ASK1 signaling cascade and ultimately the cellular response

ASK1 activity, it was demonstrated that mutation of ASK1 Cys250 to alanine (Cys250A) blocks the binding of Trx1. In this instance, ASK1 is still able to form disulfide bond-linked multimers but it is not constitutively phosphorylated on Thr838, nor can it activate downstream MAPK pathway members. These results suggest that simple dissociation of Trx1 from ASK1, while a trigger is not sufficient to activate ASK1. Further studies are required to define the regulatory role that Cys250 plays in the activation of ASK1 in response to H_2O_2 signaling.

Additional studies have demonstrated that in the absence of Trx1, tumor necrosis factor-α receptor-associated factors (TRAF) 2 and 6 are recruited to the ASK1 signalosome [14, 19, 30]. Recruitment of TRAF2 and 6 induces ASK1 phosphorylation and activation and stabilizes a higher molecular weight ASK1 signalosome complex. Consistent with this, H_2O_2-induced activation of ASK1 was strongly inhibited in mouse embyonic fibroblats (MEF's) deficient in TRAF 2 and 6 [19]. In addition, the residue Thr838 (Thr845 in mouse) located in the activation loop of the kinase is trans/auto-phosphorylated [18] and the affinity of ASK1 for its substrate MKK6, was observed to be significantly increased [31].

ASK1 is phosphorylated at multiple sites and to date, seven phosphorylation sites have been identified *in vitro*. As mentioned above phosphorylation of Thr838 located in the kinase activation loop is required for ASK1 kinase activity. In addition to ASK1 autophosphorylation, positive regulators of ASK1 activity including the family member ASK2 [32], and the murine protein serine/threonine kinase 38 (MPK38), a member of the AMP-activated protein kinase-related serine/threonine kinase family [33], were found to complex with ASK1 and to stimulate its activity by phosphorylating the Thr838 residue in response to H_2O_2 or TNF treatment. Conversely, several mechanisms negatively regulate ASK1 activity by modulating the phosphorylation status of this critical residue (Figure 3). For example, the protein phosphatase 5 (PP5), a member of the serine/threonine protein phosphatase family is reported to be recruited into the ASK1 signalosome and to dephosphorylate Thr838 subsequent to H_2O_2 treatment [34]. By dephosphorylating this critical residue, PP5 was shown to inhibit H_2O_2-induced ASK1 catalytic activity as well as ASK1-mediated apoptosis. Dephosphorylation of Thr838 inactivates ASK1 in a negative feedback manner and thereby modulates the activation of JNK/p38 and apoptosis. Such a negative feedback system most likely interplays with other cellular signal transduction pathways and is critical for determining cell fate (survival or cell death) in response to cellular stressors. Interestingly, overexpression of PP5 was shown to prevent amyloid-β-induced MAPK activation and neurotoxicity [35].

The calcium and intergrin binding protein 1 (CIB1) was also found to inhibit autophosphorylation of ASK1 at Thr838, by directly interacting with ASK1. This interaction was observed to compete with and inhibit TRAF2 recruitment to the ASK1 complex [36], repressing ASK1 activation in response to both tunicomycin, an ER stressor and 6-hydroxydopamine (6-OHDA) in dopaminergic cells. Furthermore, the block to ASK1 activation occured in a Ca^{2+}-dependent manner indicating that CIB1 functions as a Ca^{2+}-sensitive negative regulator of ASK1 activity. Thus, in an aging brain where calcium homeostasis is dysregulated [37, 38], the function of this calcium sensitive ASK1 repressor may be altered, leading to abnormal ASK1 activity and to the development of age-related neurodegenerative disorders.

ASK1 activity is further regulated by additional phosphorylation/dephosphorylation events that occur at serine-83 (Ser83), serine-967 (Ser967), serine-1034 (Ser1034), and tyrosine-718 (Tyr718). The N-terminal domain of ASK1, surrounding the Ser83 residue was

found to contain a consensus Akt phorphorylation site and biochemical and cell-based studies confirmed this site as a substrate for Akt [39]. In addition, Hsp90 was found to form a complex with Akt and ASK1 in unstimulated cells, and to stabilize the Akt-ASK1 interaction under oxidative stress conditions in order to suppress apoptosis [40]. Importantly, Akt-induced inhibition of ASK1 was observed to promote cell survival and to mediate selenite-induced neuroprotection after cerebral ischemia in rat hippocampus [41]. In a study conducted by Nakagami *et al*., activation of Akt was also observed to inhibit the toxic action of amyloid-β and to protect neurons from apoptosis [42]. These authors hypothesized that the suppression of cell death was mediated at least in part, by the ability of Akt to repress ASK1 activity. More recently, the proto-oncogene serine/threonine kinase, PIM1 was also shown to interact with and to phosphorylate ASK1 on Ser83 [43]. PIM1 phosphorylation of ASK1 decreased ASK1 activity and attenuated H_2O_2-induced ASK1 mediated activation of JNK/p38 and caspase-3. Thus, phosphorylation of ASK1 on Ser83 by Akt or PIM1 maintains ASK1 in an inactive state and suppresses ASK1-mediated p38/JNK downstream signaling.

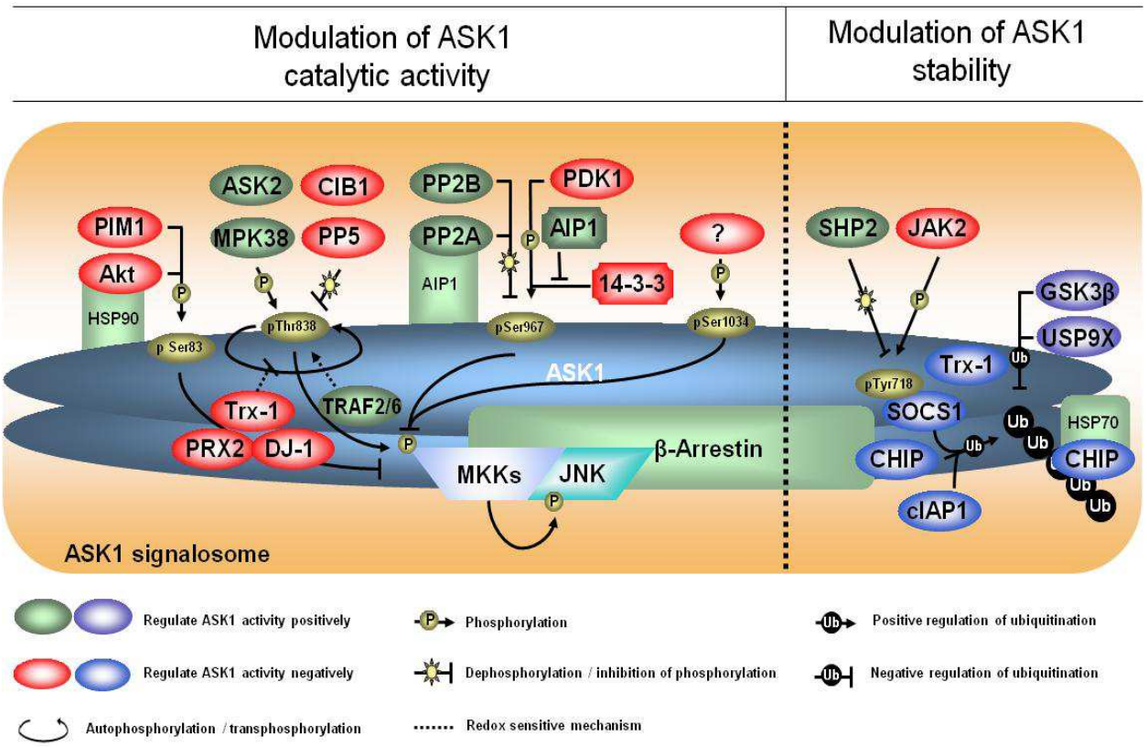

Fig. 3. Schematic representation of the multiple mechanisms regulating the catalytic activity and the stablility of ASK1 signalosome. ASK1 catalytic activity is modulated by the phosphorylation/dephosphorylation of critical residues as well as by the interaction with redox sensitive molecules. In addition, ASK1 signaling can be regulated by proteins that modulate ubiquitination of the complex and thus its stability. Green and purple represent the proteins involved in mechanisms that enhance ASK1 activity whereas red and blue represent proteins that inhibit ASK1 signaling. Together, it's the complex regulation of these mechanisms that are thought to modulate the specificity and strength of the ASK1-MKKs-JNK/p38 signaling cascade

A study conducted by Zhang *et al*., revealed that the association of ASK1 with 14-3-3 protein suppresses ASK1-mediated apoptosis [44]. These authors demonstrated that phosphorylation of Ser967, a residue located C-terminal to the ASK1 kinase domain, is critical for ASK1/14-3-3 complex formation. Interestingly, the 14-3-3 binding motif in ASK1 is conserved among its homologues from human, mouse and Drosophila, suggesting the evolutionary importance of this interaction. Importantly, exogenous expression of $ASK1^{S967A}$, a 14-3-3 defective mutant, dramatically enhanced cell death, suggesting that 14-3-3 association inhibits the death promoting activity of ASK1. More recently, Seong *et al*., demonstrated that Ser967 was phosphorylated by the 3-phosphoinositide-dependent protein kinase 1 (PDK1), a member of the protein kinase A,G, and C subfamily of protein kinases [45] and that binding of PDK1 to ASK1 was mediated through the pleckstrin homology domain of PDK1 and the C-terminal regulatory domain of ASK1. This interaction was shown to suppress H_2O_2-induced ASK1-JNK-p38 signaling as well as ASK1-mediated apoptosis. In addition, ASK1 was also observed to phosphorylate and inhibit PDK1 acitivity, suggesting a novel mechanism whereby ASK1 and PDK1 negatively regulate their respective kinase activity in a reciprocal manner [45].

Oxidative stress such as H_2O_2, was found to increase ASK1 catalytic activity by inducing dephosphorylation of ASK1 at Ser967 leading to ASK1/14-3-3 complex dissociation [46]. Two phosphatases that dephosphorylate ASK1 at Ser967 have been identified so far; Calcineurin B (protein phosphatase 2B) and protein phosphatse 2A (PP2A). Calcineurin B was found to directly interact with the ASK1 C-terminus and to dephosphorylate ASK1 at Ser967 leading to the disassociation of ASK1 from 14-3-3 proteins, ASK1 activation and enhanced cardiomyocyte apoptosis [47]. A study conducted in vascular endothelial cells demonstrated that in resting cells PP2A forms a complex with the ASK1-interacting protein (AIP1), a ras GTPase-activating protein [48]. Upon TNFα treatment the AIP1/PP2A complex was found to interact with and dephophorylate ASK1 at Ser967, leading to the dissociation of its inhibitor 14-3-3 and ASK1 activation [49]. Furthermore, Aβ was found to induce ASK1 Ser967 dephosphorylation and its dissociation from the 14-3-3 protein leading to p38 activation, and induction of the pro-apoptopic BCl-2 family member, Bax [50]. Selective inhibition of PP2A prevented the activation of this signaling cascade linking ASK1 Ser967 phosphorylation status to Aβ-induced toxicity.

Similar to phosphorylation at Ser967, phosphorylation at Ser1034, a residue contained with in the C-terminal regulatory domain of ASK1 was also found to negatively regulate its kinase and proapoptotic activity [51]. While distinct from the Akt and 14-3-3 mechanisms, candidate kinases/phosphatases implicated in the modulation of Ser1034 phosphorylation status remain to be identified.

4. Regulation of ASK1 protein levels

As outlined above, ASK1 cellular activity is tightly controlled in both a spatial and temporal fashion by distinct and multiple mechanisms (Figure 3). In addition to phosphorylation, other posttranslational modifications such as ubiquitination have been observed to play an important role in regulating ASK1 activity. Ubiquitination, is a reversible posttranslational modification that is reciprocally regulated by E3 ubiquitin ligases and deubiquitinating enzymes (DUBs). A study conducted by Liu *et al*., demonstrated that the association of Trx with ASK1 suppresses ASK1 kinase activity not only by inhibiting N-terminal interactions between ASK1 molecules, but also by controling

ASK1 protein levels through ubiquitination and degradation via the 26S proteosome [23]. This work provided the first evidence that ASK1 protein expression is regulated by ubiquitination. Recently, our laboratory demonstrated that the duration of the ASK1 signal in response to oxidative stress is regulated by mechanisms modulating the degradation of the ASK1 signalosome [31]. LC-MS/MS analysis of the ASK1 signalosome, purified from cells treated with H_2O_2, revealed the presence of ubiquitinated ASK1 together with several proteins associated with the process of protein degradation such as the 26S proteasome regulatory subunit, ubiquitin-like modifier-activating enzyme1 and ubiquitin specific protease 9 X-linked (USP9X). USP9X belongs to the USP subfamiliy of deubiquitinating enzymes and is thought to regulate multiple cellular functions. In an earlier study conducted by Nagai *et al.*, a ubiquitin-like sequence in ASK1 responsible for USP9X recognition was identified [52]. These authors demonstrated that in response to oxidative stress, ubiquitination of ASK1 C-termini mediates the proteosomal degredation of ASK1. In addition, it was demonstrated that in complex with ASK1, USP9X cleaves ubiquitin from the C-terminus of stress induced ASK1, preventing degradation and stabilizing the activated form of ASK1. In keeping with this observation, knockdown of USP9X mediated by siRNA in HeLa cells, reduced H_2O_2 induced JNK and p38 activation equivalent to that observed in ASK1 deficient cells. H_2O_2-induced ASK1 activity is therefore regulated by a complex mechanism involving a balance between phosphorylation/dephosphorylation and ubiquitination levels, whereby ubiquitin dependent regulation of ASK1 is closely coupled to its activity. In this regard these authors postulated that USP9X may be a key regulator that fine-tunes the ASK1-dependent signaling cascade. Recently, Zhang *et al.* characterized region specific protein level changes in the brains of mice treated with the neurotoxin MPTP [53]. In comparison to normal brain, USP9X was significantly upregulated within the striatum, cerebellum and cortex of the MPTP treated mice, raising the possibility of a role for USP9X in neurodegeneration.

Several additional reports have emerged describing the ubiquitin dependent regulation of ASK1. It is well established that activation of the TNF receptor 2 (TNFR2) leads to activation of ASK1 and duration of TNFR2 mediated ASK1 signaling is proposed to be controled by ubiquitin-dependent proteosomal degradation of ASK1 [54]. This mechanism was shown to involve the ubiquitin protein ligase activity of the cellular inhibitor of apoptosis protein 1 (cIAP1) and genetic knockdown experiments confirmed that cIAP1 was critical for limiting TNFR2 mediated p38 and JNK activation. Moreover, in a model of glaucoma, a neurodegenerative disease leading to impaired visual function, Kisiswa *et al.* observed an age dependent down-regulation of cIAP1 accompanied by accumulation of TRAF2 in the retinal ganglion cell layer [55]. Interestingly, dysregulated ASK1 activity was recently reported to be involved in glaucoma, indicating that interrupting ASK1 dependent pathways may be beneficial in the treatment of this pathology [56]. In addition to identifying USP9X in H_2O_2 induced ASK1 signalosome complexes, we also confirmed the presence of Hsp70 in these complexes. Previous studies demonstrate that Hsp70 mediates ASK1 degradation by recruiting the chaperone and ubiquitin ligase CHIP (C-terminus of Hsp70-interacting protein) [57]. Hsp70 together with CHIP have been implicated in protecting cells against cellular stress that cause neurodegenerative diseases, including Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington Disease (HD), and Amyotrophic Lateral Sclerosis (ALS). The suppressor of cytokine signaling 1 (SOCS1), another protein regulating proteosomal degradation, was also reported to interact with ASK1 and to mediate its degradation [58]. Phosphorylation of ASK1 Tyr718 residue by JAK2

was demonstrated to be critical for complex formation between ASK1 and SOCS1. In unstimulated cells this residue is phophorylated, allowing SOCS1 to interact with ASK1 and repress its activity by decreasing its stability. Conversely, stimulation with TNF induced SHP2-mediated dephosphorylation of Tyr718 and activation of ASK1 signaling leading to cell death. More recently, the glycogen synthase kinase-3 (GSK-3β) was found to mediate TLR4-induced ASK1 activation by increasing ASK1 stability [59].

In addition to posttranslational modifications, ASK1 protein levels may also be transcriptionally regulated via upregulation of *ASK1* gene expression. Studies have demonstrated that the E2F family of transcription factors (E2 promoter-binding factors) regulate the expression of ASK1 [60]. Suzuki *et al.*, recently found that onset of spinal and bulbar muscular atrophy (SBMA), a neurodegenerative disorder caused by a polyglutamine repeat (polyQ) expansion within the human androgen receptor correlates with aberrant E2F activation [61]. Thus, multiple posttranslational modifications and other regulatory events work in concert to govern the activity of ASK1 under both physiological and pathological conditions. The aberrant activation of ASK1 observed in neurodegenerative diseases may be triggered by multiple stimuli (e.g. oxidative stress, ER stress) by acting directly on ASK1 molecules or by impairing the activity of other proteins implicated in regulating its activity.

5. ASK1 in Alzheimer's Disease

Alzheimer's Disease (AD), the most common form of dementia, was first described by Alois Alzheimer 100 years ago. It is a neurodegenerative disorder characterized clinically by the progressive loss of memory and cognitive impairment. AD is pathologically characterized by the accumulation of cerebral neuritic plaques of amyloid-β (Aβ), neurofibrillary tangle (NFT) formation as well as by neuronal cell death. Accumulation of misfolded proteins together with increased oxidative stress and mitochondrial dysfunction are mechanisms that correlate with the pathogenesis of the disease [62, 63]. Although age is the greatest risk factor for AD, the molecular mechanisms underlying the cause of the disease remain mainly elusive. As ASK1 is activated by ROS and ER-stress (UPR), several studies have implicated ASK1 in cell death processes associated with AD. Cell-based studies including, studies employing primary hybrid neuron cells (F11, hybrid cells of rat embryonic day 13 primary cultured neurons and a mouse neuroblastoma) demonstrated that ASK1 can form a complex with the amyloid precursor protein (APP) via JIP-1b (JNK-interacting protein), phospho-MKK6 and JNK1 [65-67] resulting in caspase dependent neuronal cell death [65]. In addition, the resulting neurotoxicity was significantly blocked by exogenous expression of a dominant negative mutant form of ASK1 as well as by the JNK inhibitor SP600125 [65] strongly suggesting an ASK1/JNK-mediated death pathway in AD.

The ASK1/APP complex formation was also confirmed by three-dimensional reconstruction of confocal microscopic Z-stacks obtained from fixed brains of APP transgenic mice that revealed an up-regulation of ASK1 expression in these mice compared to non-transgenic controls [66]. Beside ROS-induced ASK1 activation, Aβ, a toxic cleavage product of APP, was also demonstrated to activate ASK1 and subsequently JNK [68]. Whereas primary neuronal cultures derived from E14.5 $ASK^{+/+}$ mice demonstrated an 80% reduction in cell viability after exposure to $A\beta_{25-35}$, the survival in $ASK^{-/-}$ derived neurons treated with $A\beta_{25-35}$ was significantly elevated (only 30% reduction in viability). Furthermore, postmortem analysis of AD patient brains compared with age-matched controls revealed strong expression of the downstream ASK1 substrate MKK6 [69]. The activated phospho-MKK6

also co-immunoprecipitated with the paired helical filament Tau from human AD hippocampal supernatants [67] and overlapped with active p38. Both are found to be exclusively localized in classic pathological AD structures like NFT and senile plaques [69]. These data strongly suggest that ASK1 could play a significant role in the pathogenesis of AD by mediating ROS and/or Aβ induced neuronal cell death via the MKK6/JNK/p38 pathway. In addition, Aβ was not only shown to cause neuron-specific toxicity, but also demonstrated to cause vascular degeneration in cerebral amyloid angiopathy; Hsu and co-workers employed primary murine cerebral endothelial cells (CEC) to investigate the mode of cell death mediated by Aβ in ASK1 transfected CECs [50]. Aβ exposure was observed to result in an induction of the ASK1-MKK3/6-p38-p53 signaling machinery and increased levels of the pro-apoptotic protein Bax resulting in CEC programmed cell death. ASK1 activity is also known to be modulated by oxidized thioredoxin-1 (TRX1) and glutaredoxin-1 (GRX1). Postmortem brain samples of AD patients revealed that whereas TRX1 was decreased in neurons (frontal cortex and hippocampal CA1 regions) GRX1 expression was increased [70]. In addition, the same authors demonstrated that in SH-SY5Y cells, $A\beta_{1-41}$ exposure resulted in TRX1/GRX1 oxidation with subsequent induction of apoptosis. Current studies therefore suggest that ER-stress and ROS-mediated ASK1 activation represents an important signal transduction mechanism in AD.

6. ASK1 in Parkinson Disease

Parkinson's Disease (PD) is the second most common neurodegenerative disease after AD. The main pathology of PD is characterized by the severe loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) that project into the striatum. PD is also characterized by the misfolding of α-synuclein which generates protein aggregates called Lewy bodies [71]. Clinical signs of PD, which include rest tremor, rigidity and bradykinesia become evident when approximately 80% of striatal dopamine and 50% of nigral neurons are lost [72]. Like AD, age is the greatest known risk factor for PD. While the mechanism underlying the 'area-specific' neuronal loss in PD remains unclear, both oxidative and ER-stress are strongly implicated as contributing factors of the disease state [73-76].

The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes the selective loss of dopaminergic neurons in SNpc in mice in a manner similar to that seen in PD and is often used in mouse models to study the molecular mechanisms in PD pathogenesis. Activation of JNK and p38 appear to be critical in mediating MPTP induced toxicity, as dopaminergic neurons in JNK3 knockout mice were significantly protected from the toxic effects of MPTP [77, 78]. The activation of JNK and p38 in dopaminergic neurons is thought to be mediated by ASK1 [79]. Karunakaran and co-workers demonstrated that administration of the neurotoxin MPTP, induced ASK1 activation and the subsequent activation of its downstream targets MKK4 and JNK [80]. This pathway triggered the nucleus to cytoplasmic translocalization of the death-associated protein Daxx, specifically in neurons located in the SNpc. Co-administration of MPTP with α-lipoic acid, a thiol antioxidant inhibited the activation of ASK1 and subsequent activation of its downstream targets [80]. Recently, peroxiredoxin2 (PRX2), an antioxidant enzyme, was demonstrated to protect against 6-OHDA induced neurotoxicity both *in vitro* and *in vivo* [27]. The proposed mechanism of inhibition was through blockade of ASK1 activation and its downstream JNK/p38 signaling pathway. Infusion of a lentiviral vector expressing a short hairpin RNA (shRNA) to specifically knock down ASK1 protein expression in the left SNpc of C57BL/6

mice was evaluated. Inhibition of ASK1 was observed to significantly attenuate the 6-OHDA induced ASK1/JNK signaling axis. Moreover, knockdown of ASK1 significantly protected against 6-OHDA induced death of dopaminergic neurons, improved motor function and significantly elevated dopamine levels in the striatum. Interestingly, immunological analysis of postmortem PD brain sections clearly indicate that active-ASK1 is frequently observed in SNpc neurons and co-localized in 33% of the cases with Lewy bodies and more than 60% of phospho-ASK1 neurons also revealed abnormal α-synuclein staining [27]. Taken together, the data demonstrate that redox sensitive molecules (e.g. PRX2) are able to modulate apoptotic pathways by influencing ASK1 activity and suggest that targeting either PRX2 or ASK1 may be a promising approach for neuroprotective intervention in PD.

7. ASK1 in Huntington's Disease

The expansion of CAG trinucleotide repeat units which encode for uninterrupted glutamine residues or (polyQ) is the underlying cause of at least nine inherited human neurodegenerative disorders including Huntington's Disease (HD), Spinobulbar Muscular Atrophy (SBMA) and several forms of spinocereballar ataxia (SCA) [81, 82]. HD is clinically characterized by abnormal involuntary movements, including chorea and dystonia, and cognitive impairment through a selective loss of neurons mainly in the basal ganglia and cerebral cortex [83]. HD is caused by a mutation in the *huntingtin* gene (abnormal CAG repeats in the open reading frame) which encodes a large protein (350 kDa) with an expanded polyglutamine (polyQ) tract. Interestingly, the number of polyglutamine repeats is correlated with the severity of symptoms and once expanded over a repeat of 40, HD occurs [84]. It has been demonstrated that the expanded polyQ repeats form intracellular cytoplasmic and/or nuclear aggregates with subsequent neurotoxic effects *in vitro*, in transgenic animals (overexpressing polyQ proteins) and in postmortem brains of polyQ disease patients. The neurotoxic insult is mediated by dysfunction of the ubiquitin proteosome function resulting in ER stress [85, 86].

As described above, the accumulation of misfolded proteins and induction of ER stress is a process that is known to activate ASK1. Thus, it was hypothesized that ASK1 could play a significant role in HD by modifying huntingtin [87], acting as a signal transducer at the protein level as well as a cell death modulator at the post-translational level [88]. Indeed, by comparison to wild type cells, neuronal cell viability derived from ASK1 knockout mice was significantly protected against cell death mediated by expression of polyQ79 (i.e. 79 glutamine repeats) [8]. In addition, neurons derived from ASK1 knockout mice, were also observed to be defective in proteasome inhibitor and ER stress-induced JNK activation. Recently, it was shown that inhibition of ASK1 through administration of ASK1 antibodies using a micro-osmotic pump reduces ER stress and toxicity in a HD mouse model. In addition, nuclear translocation of huntington fragments was observed in cells harboring active ASK1 enzyme, whereas inactivated ASK1-bound huntingtin prevented its nuclear translocation and improved motor dysfunction in mice [89]. Similarly, ASK1 protein levels are also increased in the striatum following injection of 3-nitropropionic acid (3-NP), a mitochondrial toxin producing age-dependent oxidative stress and a model of Huntington's Disease [90], whereas reduction of ASK1 expression using siRNA was accompanied by a reduction in cell death. Therefore, regulating the activity of ASK1 by small molecule inhibitors and/or antibodies could also reveal promising treatment strategies for HD.

8. ASK1 in Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a late onset neurodegenerative disorder, characterized by a selective loss of motor neurons in the spinal cord, brain stem and cerebral cortex [89]. Although the precise mechanism(s) for the pathogenesis of ALS remains unclear, studies highlight oxidative stress, excitotoxicity, protein aggregation and ER-stress as culprits in motor neuron demise. About 10% of ALS cases are familial (FALS) and about 1-2% are caused by mutations in the Cu/Zn-superoxide dismutase (SOD1) gene. SOD1$^{-/-}$ mice show no ALS phenotypes, indicating that the gain of function mutations in SOD1 (and over 120 have been identified in FALS) is critical for the death of motor neurons and FALS.
At the cellular level mutant SOD1 was suggested to play several roles in the pathogenesis of ALS, such as triggering abnormal protein interactions and activating caspases [92] [93]. Studies in transgenic mice expressing the ALS-linked SOD1 mutants showed an increase in the number of motor neurons with activated ASK1 and p38, strongly suggesting that ASK1-p38 pathway may be involved in neuronal cell death in FALS [94, 95]. Furthermore, several groups have demonstrated both *in vitro* and *in vivo* that the activation of ASK1 and/or its downstream pathways are associated with a selective motor neuron loss induced by the mutant SOD1 [96, 97]. Taken together these studies suggest a functional link between ER-stress and ASK1/p38 signaling axis in FALS. In a recent study by Nishitoh *et al.*, a specific interaction of SOD-1 and Derlin-1, a protein of the ER-associated degradation (ERAD) complex was observed to trigger ER-stress through dysfunction of ERAD [98]. The resulting ERAD dysfunction promoted ASK1 activation and subsequent apoptosis. These authors demonstrated that motor neuron death could be significantly reduced by the forced dissociation of mutant SOD1 from Derlin-1. Additional *in vivo* experiments revealed that deletion of ASK1 reduced the motor neuron loss and prolonged the lifespan of mutant SOD1 transgenic mice. These results suggest that Derlin-1/SOD-1 interaction promotes ER-stress, ASK1/p38 activation resulting in motor neuron cell death, a mechanism that is a key component of the disease progression of familial ALS [98].

9. ASK1 in ischemic brain injury

Ischemic brain injury is an acute or chronic disorder induced by insufficient blood flow into the brain. Hypoxic, or in the case of no oxygen supply, anoxic conditions trigger the induction of complex and overlapping signaling pathways, leading ultimately to neuronal cell death. Experimental models have demonstrated the involvement of pathways involving excitotoxicity, ionic imbalance, oxidative and nitrosactive stress resulting in neuronal cell death [99]. The activation of JNK was demonstrated in a murine model of transient focal ischemia to be a crucial signaling component mediating neuronal cell death [100] and strong evidence indicating a potential role for ASK1 in the pathogenesis of ischemic brain injury has emerged [101]. Using a cerebral ischemia rat model as well as in an *in vitro* kinase assay, ASK1 exhibited increased autophosphorylation and activity at various time points after the induction of cerebral ischemia. Furthermore, ASK1 autophosphorylation and activity were inhibited by the pre-administration of the antioxidant N-acetylcystein. Thus, activation of ASK1 may play a significant role in the apoptotic pathway following cerebral ischemia.
The heat shock protein-27 (Hsp27) was observed to be upregulated in cells surviving ischemic insults [102] and in ischemic preconditioning models [103], suggesting that Hsp-27 is associated with pro-survival cascades. Recently, studies overexpressing human Hsp-27 by viral mediated transfer and in Hsp27 transgenic mice demonstrated that Hsp-27 promoted

long-term neuroprotection against cerebral ischemia, as measured by infarct volume and sensory motor function. In addition, improvement in postischemic neurobehavioral recovery was also observed up to three weeks following cerebral ischemia [104]. At the molecular level, Hsp-27 was demonstrated to physically interact with ASK1 resulting in inhibition of ASK1 activity. Subsequent genetic knockdown of ASK1 or inhibition of the ASK1/MKK4 cascade also effectively abolished neuronal ischemia. Hsp-27 mediated inhibition of the pro-apoptotic ASK1 pathway may be a promising novel neuroprotective strategy for stroke.

10. Conclusion

Aberrant regulation of ASK1 activity is observed in a variety of neurodegenerative stress associated diseases and genetic knockout studies have delivered a strong case for ASK1 as a candidate therapeutic target in the treatment of such disorders. While ASK1 inhibitors have been claimed in the patent literature, no small molecule ASK1 inhibitors have obtained sufficient optimization characteristics for candidate selection and approval for first time in human studies. As such, there is little data available in the peer-reviewed literature concerning these inhibitors. In addition to their potential as future therapeutics, there is little doubt that small molecule inhibitors targeting ASK1 would be highly useful assets to facilitate understanding of the complex biology of ASK1. To rely on a molecular probe to make firm mechanistic conclusions about ASK1's role in cellular signaling, the selectivity of the final compound must be devoid of off-target activity. Recent characterization of the structure of the ASK1 kinase domain may facilitate development of ASK1-specific inhibitors [17]. Interestingly, Bunkoczi *et al.* observed that apart from its closely related isoform (ASK2), the nearest phylogenic neighbor to ASK1 shares sequence identity of only 50% within the kinase domain. In this regard, ASK1 may form a chemically diverse catalytic domain, which may allow a high level of kinase selectivity even with an inhibitor with an ATP competitive mode of action. Our laboratory has recently developed a biochemical assay using the full length ASK1 signalosome complex and full length substrate to identify inhibitors that are not only ATP competitive, but also substrate competitive and non-competitive with respect to ATP or substrate [31]. Since several ASK1 interacting proteins have been shown to modulate the activity of ASK1 within the signalosome complex, identification of cell permeable peptide inhibitors or development of ATP-noncompetative small molecule inhibitors that alter conformation or block ASK1 regultory protein interactions could serve as highly specific probes for ASK1. Regardless of the approach taken, careful analysis of the first generation of ASK1 inhibitors will be needed to define both the benefits and potential liabilities of mechanistically inhibiting ASK1 in cellular and animal systems.

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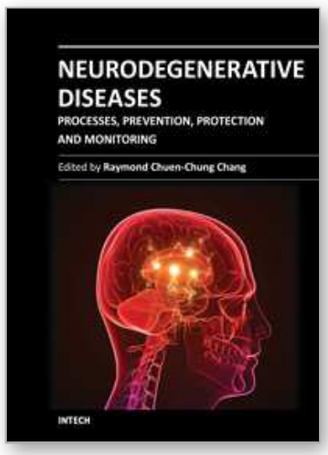

Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring

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Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring focuses on biological mechanisms, prevention, neuroprotection and even monitoring of disease progression. This book emphasizes the general biological processes of neurodegeneration in different neurodegenerative diseases. Although the primary etiology for different neurodegenerative diseases is different, there is a high level of similarity in the disease processes. The first three sections introduce how toxic proteins, intracellular calcium and oxidative stress affect different biological signaling pathways or molecular machineries to inform neurons to undergo degeneration. A section discusses how neighboring glial cells modulate or promote neurodegeneration. In the next section an evaluation is given of how hormonal and metabolic control modulate disease progression, which is followed by a section exploring some preventive methods using natural products and new pharmacological targets. We also explore how medical devices facilitate patient monitoring. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

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