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The ASK1-MAP Kinase Cascades in Mammalian Stress Response

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The mitogen-activated protein (MAP) kinase cascades play essential roles in a variety of cell processes by influencing transcriptional or translational regulation. ERKs play a central role in survival and mitogenic signaling, while JNKs and p38 MAP kinases are preferentially activated by environmental stresses and are actively involved in various stress responses including cell death, survival and differentiation. Apoptosis signal–regulating kinase 1 (ASK1)—a serine/threonine protein kinase—is a member of the MAPKKK family and activates both JNK and p38 pathways. It is well known that ASK1 is activated in cells treated with death receptor ligands and oxidant stress, such as that caused by hydrogen peroxide (H_2O_2). Moreover, recent studies have revealed new mechanisms by which ASK1 is activated in response to various types of extracellular and intracellular signals, such as endoplasmic reticulum (ER) stress, calcium signaling, and G-protein coupled receptor (GPCR) signaling. This review summarizes the regulatory mechanisms of ASK1 activity and the physiological roles of ASK1-mediated signal transduction.

Key words: apoptosis/ASK1/JNK/MAPKKK/p38.

The mitogen-activated protein (MAP) kinase cascades are among the most extensively studied of the signaling systems that transmit stimuli from outside the cell to the nucleus. MAPKs play important roles in a variety of cell processes by controlling transcriptional or translational regulation (*1*, *2*). Three major MAP kinase cascades have been well characterized in mammals, converging on ERKs, c-Jun N-terminal kinases (JNKs), and p38 MAP kinases; each consists of three classes of serine/threonine kinases, MAP kinase, MAP kinase kinase (MAPKK, also referred to as MEK), and MAPKK kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, and activated MAPKK in turn phosphorylates and activates MAP kinase. Among the three MAP kinase cascades, ERKs are activated by various cytokines and growth factors and play a central role in cell growth and differentiation. On the other hand, JNKs and p38 MAP kinases are preferentially activated by chemical and physical stressors such as UV radiation, X-ray, heat shock and osmotic shock, and by proinflammatory cytokines such as tumor necrosis factor (TNFα), and these cascades control stress adaptation, cell death and survival.

Apoptosis signal–regulating kinase 1 (ASK1), a 160-kDa serine/threonine protein kinase, is a member of the MAPKKK family and activates both p38 and JNK pathways by directly phosphorylating and activating SEK1 (also known as MKK4)/MKK7 and MKK3/MKK6 (*3*, *4*). ASK1 is activated in cells treated with death receptor ligands such as TNFα and Fas ligand. ASK1 is also activated by various forms of cytotoxic stress, such as that induced by hydrogen peroxide (H_2O_2), anticancer drugs, and growth factor deprivation (*5*–*7*). Furthermore, recent studies have revealed new mechanisms by which ASK1 is activated in response to a variety of signals; endoplasmic reticulum (ER) stress, calcium signaling, and G-protein coupled receptor (GPCR) signaling (*8*–*10*). Overexpression of wild-type ASK1 or the constitutively active mutant causes mitochondria-dependent apoptosis by releasing cytochrome *c* and activating caspase-3/caspase-9 in various cell types (*6*, *7*, *11*). In this cascade, Yamamoto *et al.* demonstrated that ASK1-mediated JNK activation phosphorylates Bcl-2, leading to the reduction of its anti-apoptotic activity (*12*). However, the detailed molecular mechanisms that link mitochondria-dependent apoptosis and ASK1-p38/JNK activation remain unknown.

On the other hand, overexpression of ASK1 induces not only apoptosis but also cell differentiation and survival, depending on the cell types and cellular context analyzed; ASK1 induces neurite outgrowth in PC12 cells, and keratinocyte differentiation, predominantly by activation of the p38 pathway (*13*, *14*). In this review, we discuss the activation mechanisms of ASK1 and the physiological roles of ASK1-mediated signal transduction.

Structure and activation mechanisms of ASK1

Human and mouse ASK1 consist of 1,374 and 1,379 amino acids, respectively, and each has a serine/threonine kinase domain in the middle of the molecule (*4*). ASK1 has two coiled-coil domains (N-terminal and C-terminal), and an FKBP-type peptidyl-prolyl *cis-trans* isomerase motif in the N-terminal (the function of which is unknown). 14-3-3 proteins bind to the C-terminal 14-3-3 binding motif and suppress ASK1 activity (Fig. 1; see below). The mechanism of ASK1 activation involves homo-oligomerization. It was demonstrated that synthetic ASK1-ASK1 fusion constructs activate JNK and p38 pathways (*15*, *16*). ASK1 in the resting state constitutively forms a homo-oligomer through its C-terminal coiled-coil domain. Upon stimulation with H_2O_2, additional interface appears to be created on the pre-formed

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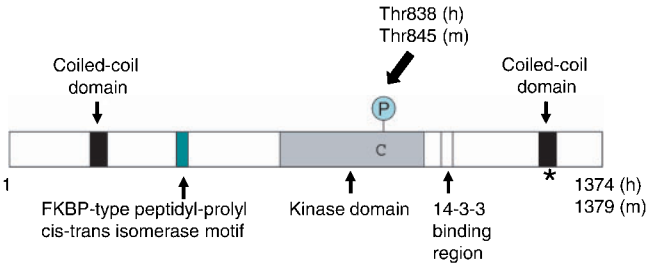

Fig. 1. **Schematic representation of ASK1 protein.**

ASK1 oligomer, which leads to autophosphorylation of Thr845 in mouse ASK1 (Thr838 in human ASK1) (*16*). This threonine residue is located in the so-called activation loop of the kinase domain. As the kinase activity of the alanine-exchange mutant of this residue (T845A) is strongly reduced in response to H_2O_2 stimulation, phosphorylation of Thr845 was shown to be essential for ASK1 activation; the specific antibody that recognizes phospho-Thr845 has thus been a useful indicator of activated ASK1 (*9*, *16*, *17*).

Oxidative stress and ASK1-interacting proteins

Reactive oxygen species (ROS), such as superoxide anions and H_2O_2, which are produced through a variety of cellular processes or derived from exogenous sources, play important roles in the regulation of normal physiology, including cell proliferation, survival, and immune responses (*18*). However, excessive production of ROS contributes to severe damage to cellular components, loss of cell function, and ultimately apoptosis or necrosis. For example, it is well established that excess ROS contributes to processes such as heart failure, myocardial infarction, and neuronal cell death (*19*, *20*). ASK1 is strongly activated in cells exposed to various oxidants and is involved in oxidative stress–induced apoptosis (*7*, *21*, *22*).

Several signaling molecules have been found to negatively regulate the oxidant stress-induced ASK1 activation. The ASK1 repressor thioredoxin (Trx) was initially identified from a yeast two-hybrid screen for ASK1-binding proteins (*7*). Trx, a ubiquitously expressed reduction/oxidation (redox)–regulatory protein, directly binds to the N-terminal region of ASK1 and somehow inhibits its kinase activity (Fig. 2). The binding of Trx to ASK1 requires the presence of a reduced form of an intramolecular disulfide bridge between two cysteine residues in the catalytic site of Trx, Cys32 and Cys35. An *in vitro* binding assay showed that oxidized Trx, or Trx mutants in which both of these residues have been mutated, could not bind to or inhibit ASK1. Upon H_2O_2 stimulation of HEK293 cells, oxidized Trx is dissociated from ASK1, and the freed ASK1 is activated by formation of an oligomeric complex, followed by Thr845 autophosphorylation. This Trx-ASK1 binding system is also involved in TNFα signaling, in that TNFα-induced intracellular ROS appears to dissociate Trx from ASK1 (*7*).

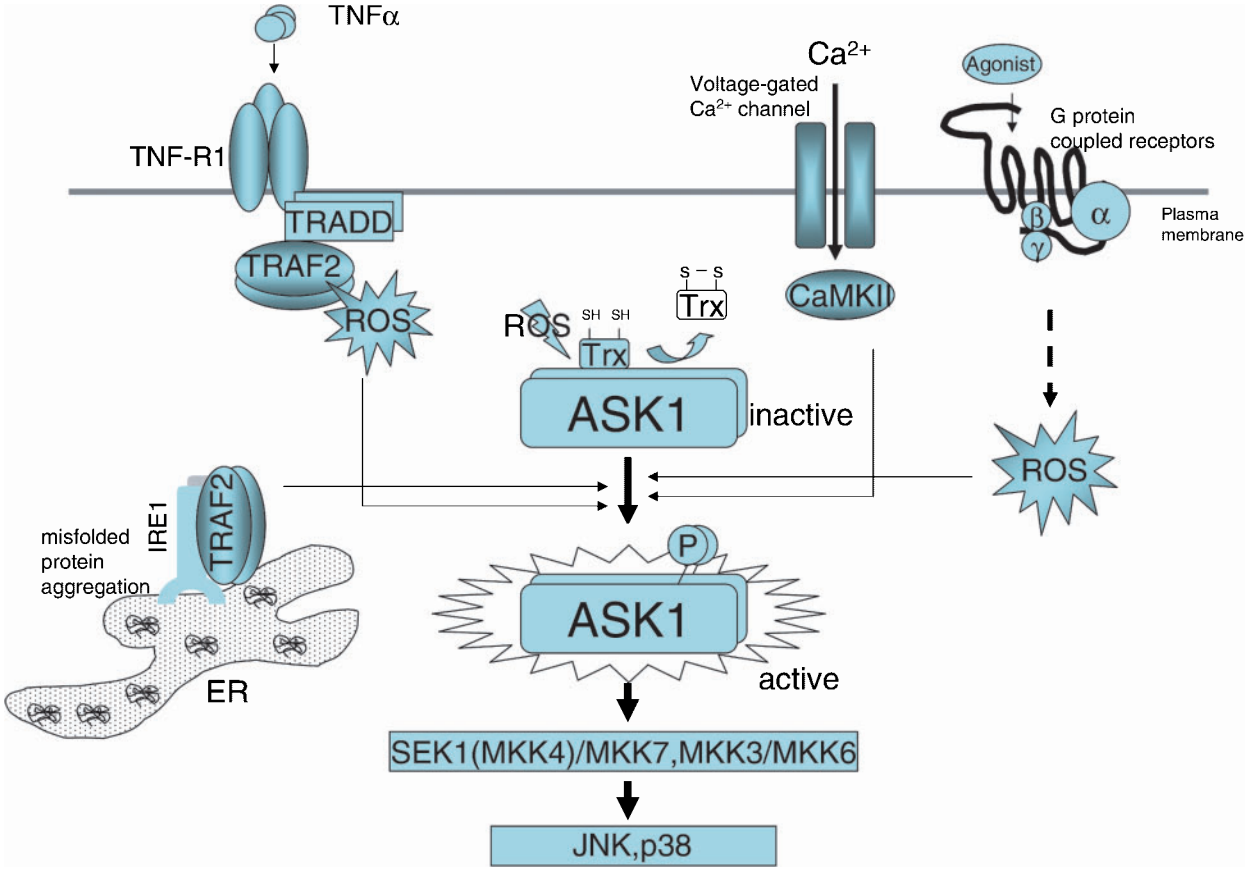

Fig. 2. **Regulatory mechanisms of ASK1 in response to various stresses.**

Recently, other signaling molecules that directly bind to ASK1 have been shown to regulate oxidative stress-induced ASK1 activation. Glutaredoxin, another intracellular redox-signaling molecule, inhibits glucose deprivation-induced ASK1 activation (a form of metabolic oxidative stress) (*23*). Protein serine/threonine phosphatase 5 (PP5) directly dephosphorylates Thr845 and inactivates its kinase activity both *in vitro* and *in vivo* (*17*). As the interaction between PP5 and ASK1 is induced by H_2O_2 stimulation, it seems to operate as a negative feedback system of ASK1 activation. The 14-3-3 proteins, phosphoserine/phosphothreonine–binding molecules, bind to ASK1 through phosphorylated Ser967 (located in the 14-3-3 binding motif of ASK1 and phosphorylated in the resting state) and suppress ASK1-induced apoptosis (*24*). Goldman *et al.* demonstrated that H_2O_2 induces Ser967 dephosphorylation, and that this dephosphorylation correlates with increased ASK1 activation (*25*). These data suggest that 14-3-3 may also act as a negative regulator of ASK1 activation. However, the Ser967-specific phosphatase and kinase involved have yet to be identified.

To confirm the necessity of ASK1 activation in oxidative stress-induced apoptosis, we generated and analyzed ASK1-null mice (*22*). ASK1–/– mice were born at the expected Mendelian frequency and did not differ significantly in appearance from the wild type. Mouse embryonic fibroblasts (MEFs) isolated from ASK1–/– mice were resistant to H_2O_2-induced apoptosis. Moreover, in ASK1–/– MEFs, H_2O_2-induced sustained, but not transient, JNK and p38 activation was suppressed. These findings indicate that the ASK1-p38/JNK cascade is essential for H_2O_2-induced apoptosis, and that multiple molecules are coordinately involved in ASK1 regulation.

Death receptor–mediated ASK1 activation

Previous studies indicate that ASK1 is required for JNK/p38 activation induced by various death-receptor ligands (*4*, *22*, *26*). Fas, a well-characterized member of the death receptor family, assembles a signaling complex called DISC (death-inducing signaling complex) upon activation by FasL or agonistic antibodies. This complex contains the death domain–containing adaptor protein FADD (Fas-associated death domain protein) and caspase 8. The formation of DISC leads to the acute execution of apoptosis (*27*). On the other hand, there is a second FADD/caspase 8–independent pathway, which leads to JNK activation. This alternative pathway is initiated by the interaction of Fas with the receptor-associated protein Daxx. Upon Fas activation, Daxx binds to the N-terminal of ASK1 and subsequently activates JNK, which may sensitize cells to apoptosis (*26*). However, mutant Fas that is incapable of recruiting FADD but still interacts with Daxx, does not cause apoptosis, despite still being able to activate JNK (*28*). Moreover, the sensitivity to Fas-induced apoptosis was indistinguishable between ASK1+/+ and ASK1–/– thymocytes despite the abolition of Fas-dependent JNK/p38 activation in ASK1–/– cells (*22*). These data indicate that the Daxx-ASK1-JNK/p38 pathway is not essential for Fas-induced apoptosis, at least in thymocytes, and suggest that this pathway may have unknown physiological roles in Fas signaling.

The proinflammatory cytokine TNFα regulates immune responses, inflammation, and apoptosis. TNFα is also a strong activator of ASK1, and this activation is regulated by TNF receptor–associated factor 2 (TRAF2) (*29*). TRAF2 is an adaptor protein that couples TNFα receptors, and overexpression of TRAF2 activates the ASK1-JNK/p38 cascade. Moreover, TRAF2 directly interacts with the C-terminal domain of ASK1 upon TNFα stimulation (*30*). The regulation of ASK1 by TRAF2 is redox-dependent. TNFα induces the dissociation of Trx from ASK1, an effect that is sensitive to pretreatment with antioxidants (*7*). Moreover, it has been reported that TRAF2 overexpression or TNFα treatment leads to the production of ROS (*30*, *31*). This suggests that Trx acts as a negative regulator of TNFα-induced ASK1 activation as well as H_2O_2-induced activation. Furthermore, MEFs derived from ASK1–/– mice are significantly resistant to TNFα-induced apoptosis, and the sustained activation of JNK and p38 is reduced, indicating that ASK1 is required for TNFα-induced apoptosis (*22*). In fact, sustained activation of JNK/p38 by TNFα stimulation has been implicated in the apoptosis of several cell types (*32*–*34*).

ER-stress–induced apoptosis and ASK1 activation

The accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER) induces the unfolded protein response (UPR). The network of physiological responses to UPR is regulated by ER transmembrane proteins: IRE1, PERK, and ATF6. PERK and IRE1 are ER-resident type I transmembrane serine/threonine protein kinases, and are autophosphorylated and activated in response to the accumulation of unfolded proteins. In contrast, ATF6 is a basic leucine-zipper transcription factor cleaved and activated in the Golgi apparatus, and functions as a cytoplasmic transcription factor. Activation of these molecules leads to a reduction in the influx of nascent protein into the ER. If these adaptive responses are not sufficient to relieve ER stress, the cell undergoes apoptosis. For example, IRE1 activates the JNK-signaling pathway mediated by TRAF2 in response to excess ER stress (*35*).

ER stress–induced apoptosis is linked to human pathogenic conditions such as amyloidosis, hypercholesterolemia, diabetes, and neurodegenerative disorders (*36*, *37*). For example, polyglutamine diseases (*e.g.* Huntington's disease) are neurodegenerative diseases caused by the expansion of a CAG repeat in the disease gene. The CAG repeats code for an expanded polyglutamine (polyQ) tract in the protein (*38*, *39*). Although several studies suggest that polyQ exerts its toxic effect by forming insoluble intracellular aggregates, the molecular mechanisms involved remain unknown. Recently, we have shown that abnormally long polyQ fragments trigger ER stress and subsequent ASK1 activation (*8*). In PC12 cells, polyQ activates IRE1, which recruits TRAF2. ASK1 interacts directly with TRAF2 and activates the SEK1-JNK pathway. The polyQ-dependent activation of the ASK1-SEK1-JNK cascade and neuronal apoptotic cell death were considerably suppressed in ASK1–/– primary neurons. Moreover, in ASK1–/– primary neurons, proteasome inhibitor (MG132)–induced cell death was also reduced. These results indicate that ASK1 is required for polyQ-

dependent neuronal cell death, and also suggest that this polyQ-dependent ER stress occurs in part through proteasome dysfunction. Indeed, several groups have reported that the proteasome activity is inhibited in polyQ-expanded huntingtin (*40–42*).

G-protein coupled receptor (GPCR) signaling and ASK1 activation in cardiomyocytes

McDonald *et al*. originally reported G-protein coupled receptor (GPCR)–mediated ASK1 activation (*10*). They found that β-arrestin 2, a key molecule for receptor desensitization and internalization, also functions as a scaffold protein for the ASK1-SEK1-JNK3 cascade, and that these complexes are related to Angiotensin II-induced JNK3 activation (*43*). However, the physiological role of this system has remained unknown. Recently, it has become apparent that G-protein coupled receptor (GPCR)–mediated ASK1 activation is closely related to the intracellular production of ROS and cardiac dysfunction. Izumiya *et al.* demonstrated that ASK1 is essential for angiotensin II–induced cardiac hypertrophy by using ASK1–/– mice. In ASK1–/– mice, angiotensin II–induced left ventricular (LV) superoxide production and JNK/p38 activation were remarkably reduced compared to wild-type mice (*43*). Moreover, angiotensin II–induced cardiac hypertrophy and remodeling were significantly reduced in ASK1–/– mice. These data suggest that ROS induced by physiological GPCR agonists activate the ASK1-JNK/p38 pathway, which is responsible for the hypertrophic response in cardiomyocytes. Although the relationship between arrestin-dependent activation and ROS-dependent activation of ASK1 remains uncertain, ASK1 seems to be a promising therapeutic target for cardiac dysfunction.

Calcium signaling and ASK1-p38 cascade activation

Calcium plays important roles in regulating a variety of neuronal functions, and calcium-dependent activation of MAPK is involved in processes such as synaptic plasticity (*44*). For example, calcium enters neurons via NMDA receptors or voltage-gated calcium channels, binds to calmodulin (CaM), and activates the ERK pathway. In this cascade, CaM-binding proteins (CaMBPs), such as Ras-GRF (neuron-specific GEF for Ras) and CaM-dependent protein kinase IV (CaMKIV), positively modulate ERK1/2 activation induced by NGF or membrane depolarization. However, the relationship between calcium signaling and JNK/p38 activation in neuronal processes has not been well defined. Sagasti *et al.* reported that the CaMKII-ASK1-p38 axis operates developmentally to induce asymmetric expression of a certain type of odorant receptor in nematode (*45*). Recently, we have shown that calcium signaling molecules regulate the ASK1-p38 MAPK cascade in mammalian neurons (*9*). In primary neurons from ASK1+/+ mice, potassium depolarization, which induces extracellular calcium influx, activated the ASK1-p38 cascade, and this activation was impaired in primary neurons derived from ASK1–/– mice. The activation of ASK1 induced by calcium influx was suppressed by KN-93 (CaMKs inhibitor) and by siRNA-mediated reduction of CaMKII expression. Furthermore, an *in vitro* kinase assay showed that CaMKII phosphorylates ASK1, and these two kinases form a molecular complex in HEK293 cells. These results indicate that the CaMKII-ASK1-p38 MAP kinase cascade constitutes a novel calcium signal mediator in neurons.

Conclusion

This review focused on the regulation of ASK1 and its physiological role in mammalian cells. ASK1 causes apoptosis in response to common pro-apoptotic stresses, such as oxidative stress, and death receptor ligands. Moreover, pathogenic stress (ER stress, GPCR-induced ROS production) also causes apoptotic cell death through activation of ASK1-JNK/p38 cascades in neurons and cardiomyocytes. These data suggest that ASK1 may be a promising therapeutic target for treatment of neurodegenerative disorders and cardiac dysfunction. On the other hand, in PC12 cells and keratinocytes, ASK1 induces differentiation and survival via p38 activation. Furthermore, CaMKII phosphorylates ASK1 and activates the ASK1-p38 pathway in neurons, and this p38 activation may play important roles in synaptic plasticity. Thus, ASK1 is not only able to induce apoptosis, but also mediates a wide range of cellular functions. Further knowledge of its regulatory mechanisms may make ASK1 a more promising therapeutic target for apoptosis-based incurable diseases. In addition, an understanding of the novel physiological roles of ASK1 may shed light on the diverse cellular processes regulated by this important protein kinase.

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