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Obligatory role of ASK1 in the apoptotic surge of K⁺ currents

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Abstract

Apoptosis signal-regulating kinase 1 (ASK1) is a critical component of mitogen-activated protein kinase signaling pathways leading to cell death in response to cytokines and cellular stress. We use a dominant-negative (DN) form of ASK1 to show that this enzyme is necessary for the delayed surge in neuronal K⁺ channel activity, a required step in apoptosis. Furthermore, expression of ASK1 DN also suppresses the apoptotic increase in Kv2.1 currents transiently expressed in Chinese hamster ovary cells. Finally, over-expression of thioredoxin, an inhibitory binding partner of ASK1, is sufficient to halt the apoptotic current surge in neurons. Thus, ASK1 is an obligatory component of the pro-apoptotic modulation of K⁺ channels.

Keywords

Apoptosis signal-regulating kinase 1; Potassium channels; Oxidative stress; Cortical neuron; Thioredoxin

Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed mammalian mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) that activates both the p38 and c-JUN NH₂-terminal kinase (JNK) by directly phosphorylating their respective MAPKKs (MKK3/6 for p38 and MKK4/7 for JNK) [13]. ASK1 has been shown to be an important signaling kinase in apoptotic cell death in response to various stimuli, including oxidative stress, tumor necrosis factor, endoplasmic reticulum stress, low-potassium, and amyloid β [7, 9,10,15,27,28]. Although ASK1 activation has been repeatedly linked to neuronal apoptosis [9,15,20,28], the specific downstream molecular cascades leading to cell death in these systems have yet to be fully characterized.

Cellular potassium efflux is a common, required feature in many apoptotic programs [6,29]. In cortical neurons, the loss of intracellular potassium occurs through a delayed surge in voltage-dependent K⁺ currents [32] that is mediated by Kv2.1-encoded ion channels [16]. This increase in K⁺ currents can be triggered by classical apoptogens, such as staurosporine, serum deprivation, and amyloid β [30–32], as well as by oxidants like 2,2'-dithiodipyridine (DTDP) [1,14] and peroxynitrite [2,17]. We have previously observed that the apoptotic K⁺ current surge is protein synthesis independent and precedes caspase activation [14]. Importantly, activation of the MAPKp38 is required for this process as both chemical inhibitors of this enzyme [14] and expression of p38 dominant negative (DN) vectors [2,17] can completely abolish the increase in K⁺ currents.

In addition to ASK1, p38 activation can be induced by other MAPKKK, including transforming growth factor- β -activated kinase 1 (TAK1), mixed-lineage protein kinase 3 (MLK3) and

mitogen-activated protein three kinase 1 (MTK1) [33]. Furthermore, it has been reported that p38 can also be activated via a MAPKKK-independent mechanism [4]. We report here that ASK1 represents the principal, if not the only, upstream signaling MAP kinase linking oxidative injury to the apoptotic increase in Kv2.1-mediated currents in neurons.

Experiments were performed on cultures of embryonic rat cerebral cortex and on Chinese hamster ovary (CHO) cells. Cortical cultures were prepared from embryonic day 16 Sprague–Dawley rats and grown on 12mm glass coverslips as previously described [5]. Pregnant rats were sacrificed with the approval of the University of Pittsburgh School of Medicine and in accordance with National Institutes of Health protocols. Enhanced green fluorescent protein (eGFP) (pCMVIE-eGFP; Clontech, Palo Alto, CA, USA) was used as a marker for positively transfected neurons and was combined with the ASK1 DN or Trx cDNAs (gifts from H. Ichijo, Tokyo, Japan), or vector, at a 1:1 ratio. At DIV19–23, cortical cultures were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) [16]. Briefly, 1.5 µg of cDNA was diluted in 50 µl Opti-Mem I medium and combined with 50 µl of Opti-Mem I medium containing 2 µl Lipofectamine 2000. Complexes were allowed to form for 30 min before addition to the cultures. Cortical cells were maintained for 48 h at 37 °C, 5% CO₂ before electrophysiological recordings. Chinese hamster ovary cells were seeded at 2.8×10^5 cells per well into six-well plates 24 h before transfection. Cells were transfected with a Kv2.1eGFP-myc tagged cDNA plasmid (a gift from E. Levitan, Pittsburgh, PA, USA) and ASK1 DN, or vector, at a 1:1 ratio in serum-free medium with 6 µl of Lipofectamine reagent (Invitrogen), and a total of 1.4 µg of DNA per well. Electrophysiological recordings from eGFP-positive CHO cells were performed 24 h after transfection. All recordings were performed using the whole-cell configuration of the patch-clamp technique as described previously [14]. The extracellular solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 MgCl₂, 10 HEPES, 10 D-glucose; pH was adjusted to 7.2 with concentrated KOH; 0.25 µM TTX was added to inhibit voltage-gated sodium channels. The intracellular (electrode) solution contained (in mM): 100 K-gluconate, 11 EGTA, 10 KCl, 1 MgCl₂, 1 CaCl₂·2H₂O, 10 HEPES; pH was adjusted to 7.2 with concentrated KOH; 0.22 mM ATP was added and osmolarity was adjusted to 280 mOsm with sucrose. All measurements were obtained under voltage clamp with an Axopatch 1C amplifier (Axon Instruments, Foster City, CA, USA) and pClamp software (Axon Instruments) using 2 MΩ electrodes. Partial compensation (80%) for series resistance was performed in all instances. Currents were filtered at 2 kHz and digitized at 10 kHz (Digidata; Axon Instruments). Potassium currents were evoked with a series of 80 ms voltage steps from a holding potential of –70 to +35 mV, in 15 mV increments. Steady-state amplitudes were measured relative to baseline and normalized to cell capacitance.

Neurons were treated with DTDP (100 µM) for 10 min at 37 °C, 5% CO₂, a condition that induces a well-characterized p38-dependent increase in K⁺ currents approximately 3 h later, followed by caspase activation at 5–7 h and subsequent cell death [14,16]. Kv2.1-expressing CHO cells were co-treated with DTDP (25 µM) and Boc-aspartate-fmk (BAF, 10 µM), a broad-spectrum cysteine protease inhibitor, for 5 min in 37 °C, 5% CO₂. Subsequently, CHO cells were maintained in BAF (10 µM) for 3 h. We have previously reported that BAF prevents Kv2.1-expressing CHO cells from dying following DTDP exposure [16], and here we found the caspase inhibitor necessary to maintain cells sufficiently healthy for successful electrophysiological recordings. All data are expressed as mean ± S.E.M. Current densities were analyzed using one-way ANOVA followed by a Bonferoni Multiple Comparisons Test. *P*-values less than 0.05 were considered significant.

We expressed an ASK1 DN vector in cortical neurons to investigate the role of this MAPKKK in the delayed apoptotic K⁺ current surge. The ASK1 DN vector (ASK1 K709M), rendered catalytically inactive by a lysine to arginine mutation, has been well characterized in prior studies [7,8,12,28]. Control and ASK1 DN-transfected cultures were treated with either vehicle

or with 100 μ M DTDP for 10 min to induce apoptosis [1,14,16]. Electrophysiological recordings were performed from these neurons approximately 3 h later, the time it normally takes to observe a pronounced K^+ current surge [14]. DTDP-treated control neurons had very pronounced K^+ currents, when compared to vehicle-treated cells (Fig. 1A and C), reflecting the dramatic increase in channel activity that accompanies the apoptotic process [14,32]. In contrast, K^+ currents in ASK1 DN-expressing neurons were essentially identical between the vehicle and DTDP treatment groups (Fig. 1B and C), suggesting that this MAPKKK was critical for the apoptotic K^+ current surge. As a positive control, electrophysiological recordings were also performed on DTDP-treated, untransfected neurons from the same coverslip where recordings from ASK1 DN-expressing cells had been obtained. In these untransfected cells, a clear K^+ current enhancement was measured (Fig. 1C), confirming that the DTDP treatment had been effective in triggering the apoptotic program in those cultures, and reinforcing the notion that ASK1 was a critical component of the current surge process.

Functional expression of Kv2.1 in Chinese hamster ovary cells was used to firmly establish the role of ASK1 in mediating the apoptotic current surge via the channel known to be responsible for the increase in K^+ currents in neurons [16]. CHO cells do not express native Kv channels [30], but can be rendered susceptible to DTDP-induced apoptosis following the expression of Kv2.1 [16]. Electrophysiological recordings were performed from control and ASK1 DN-expressing CHO cells 3 h after a 5 min exposure to vehicle or 25 μ M DTDP. K^+ currents in control cells exposed to DTDP were substantially larger than vehicle-treated cells, without a noticeable change in the voltage-dependence of channel activation, which was determined from current–voltage relationships (Fig. 2A and C). In contrast, the K^+ currents in ASK1 DN-expressing CHO cells were virtually indistinguishable between the vehicle and DTDP-treated groups (Fig. 2B and C). These data indicate that Kv2.1 is a downstream target of the apoptotic pathway triggered by ASK1 activation, even in cells that do not normally express the channel. This strongly suggests that a fairly direct and ubiquitous pathway exists between the downstream signaling cascades activated by ASK1 and the mechanism responsible for the enhanced currents mediated by Kv2.1.

In non-stressed cells, ASK1 is bound at its N-terminal region to thioredoxin (Trx) via a protein–protein interaction, deeming the kinase inactive. Trx is a redox-regulatory protein containing two sulfhydryl groups near its catalytic center. Under cell stress, reactive oxygen species can oxidize these sulfhydryl groups, inducing the release of ASK1, which becomes activated following oligomerization and autophosphorylation [11,19]. We have previously observed that a free-radical spin trap can inhibit DTDP-mediated activation of p38 in neurons [14], suggesting a role for reactive oxygen species in the activation of the MAPK. DTDP is known to trigger neuronal apoptosis by first liberating zinc from intracellular binding proteins [1]. Zinc, in turn can generate reactive oxygen species by modulating mitochondrial function [21, 22] or activating 12-lipoxygenase [34]. We thus hypothesized that over-expression of Trx may prove sufficient to halt the DTDP-induced K^+ current surge by providing an excess of the inhibitory binding partner of ASK1. As such, electrophysiological measurements were performed from vehicle and DTDP (100 μ M, 10 min)-treated neurons previously transfected with a plasmid containing Trx. We observed virtually identical current–voltage relationships and K^+ current amplitudes (Fig. 3A and B) in both vehicle and DTDP-treated neurons expressing Trx. In contrast, recordings performed on DTDP-exposed cells not over-expressing Trx, but obtained from the same coverslip, revealed a pronounced K^+ current surge, indicating that the exposure to the apoptogen had been effective across the culture. These results indicate that over-expression of Trx, a suppressor of ASK1 activation, can halt the apoptotic increase in K^+ currents in neurons. It is possible, however, that over-expression of Trx may have other, non-specific effects, including a direct interaction with DTDP. The fact that DTDP activates p38 in a ROS-dependent fashion [14] despite the presence of many endogenous thiol groups does suggest, however, that Trx directly suppresses ASK1 function.

Consistent with reports involving oxidative injury [23,24], we have found that ASK1 is a requisite upstream signaling kinase in a critical step of neuronal apoptosis; namely, an increase in voltage-dependent K^+ currents. In important recent studies, a link was established between Ca^{2+} signaling and p38 activation in neurons via the interaction of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and ASK1 [18,22,25]. CaMKII is a well-known regulator of synaptic function [3], and p38 has recently been implicated in certain forms of synaptic plasticity [26], including an NMDA (a Ca^{2+} -permeable glutamate receptor) receptor-dependent form of long-term depression that is mediated by the internalization of glutamate receptors specific for AMPA [35]. It is thus appealing to speculate that, based on our present findings, calcium signaling may be linked in future studies to long-term changes in neuronal excitability via ASK1/p38-dependent alterations in voltage-dependent K^+ currents.

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Fig. 1.

ASK1 is required for the apoptotic K⁺ current surge in cortical neurons. (A) Whole-cell K⁺ currents were evoked by a series of voltage steps from -70 mV to +35 mV in 15 mV increments in two representative control neurons exposed to either vehicle (VEH, left) or 2,2'-dithiodipyridine (DTDP; right, 100 μ M for 10 min). Note the substantial difference in current amplitudes between the two treatment groups. Also note that the current evoked by the last voltage step (+35 mV) in the DTDP-treated neuron was beyond the ability of our amplifier to appropriately hold under voltage clamp. Scale bars 5 nA, 20 ms. (B) Similar recordings were performed from ASK1 dominant negative (DN)-expressing neurons exposed to either vehicle (left) or DTDP (middle). Note the lack of an effect of DTDP on K⁺ current amplitudes under

these circumstances. As a positive control, recordings were also performed from a DTDP-treated untransfected neuron from the same coverslip where the measurement from the ASK1 DN-expressing cell had been performed (right). The large K^+ currents present in this type of cell confirmed that DTDP had triggered the apoptotic program in the culture dish. (C) Mean $\pm$ S.E.M. current densities from vehicle- and DTDP-treated control (black bars) and ASK1 DN-expressing (green bars) neurons. The blue bar represents the current densities obtained from DTDP-treated untransfected neurons in the same coverslip containing the ASK1 DN-expressing cells. Currents were evoked with a voltage step to +5 mV from a holding potential of -70 mV and normalized to cell capacitance ($*p < 0.05$; ANOVA, Bonferroni). The voltage step to +5 mV was always under adequate voltage clamp.

Fig. 2.

Apoptotic increase in Kv2.1 currents in Chinese hamster ovary (CHO) cells requires ASK1. (A) Current–voltage (*I*–*V*) relationships of K⁺ currents evoked by a series of voltage steps from –70 mV to +35 mV in 15 mV in two representative Kv2.1-expressing CHO cells exposed to either vehicle (circles) or DTDP (25 μM, 5 min; black squares). Note the increase in the current amplitude in the DTDP-treated cell, without a change in the voltage-dependence of activation. (B) Similar measurements were performed in ASK1 DN-expressing CHO cells exposed to either vehicle (circles) or DTDP (black squares). Note the virtually indistinguishable *I*–*V* curves between the two representative cells. (C) Mean ± S.E.M. Kv2.1-mediated current densities from vehicle- and DTDP-treated control (black bars) and ASK1 DN-expressing

(green bars) CHO cells. Currents were evoked with a voltage step to +5 mV from a holding potential of -70 mV and normalized to cell capacitance (* $p < 0.05$; ANOVA, Bonferoni).

Fig. 3.

Over-expression of thioredoxin (Trx) prevents the apoptotic current surge in neurons. (A) I - V relationships of K^+ currents evoked by a series of voltage steps from -70 mV to $+35$ mV in 15 mV in representative Trx over-expressing neurons exposed to either vehicle (black triangles) or DTDP (100 μ M, 10 min; black squares). Note the very similar I - V curves in these two cells. An I - V relationship was also established from a DTDP-treated untransfected neuron from the same coverslip where the measurements from the ASK DN-expressing cell had been performed (black circles), demonstrating the current surge. (B) Mean $\pm$ S.E.M. current densities from vehicle and DTDP-treated ASK1 DN-expressing neurons (green bars). The blue bar represents the current densities obtained from DTDP-treated untransfected neurons in the same coverslip

containing the ASK1 DN-expressing cells. Currents were evoked with a voltage step to +5 mV from a holding potential of -70 mV and normalized to cell capacitance (statistically different from the Trx + groups, $^*p < 0.05$; ANOVA, Bonferoni).