

## **A Death-Promoting Role for Extracellular Signal-Regulated Kinase**

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## **Abstract**

Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), which are members of the mitogen activated protein kinase (MAPK) superfamily, have been well characterized and are known to be involved in cell survival; however, recent evidence suggests that the activation of ERK1/2 also contributes to cell death in some cell types and organs under certain conditions. For example, ERK1/2 is activated in neuronal and renal epithelial cells upon exposure to oxidative stress and toxicants and deprivation of growth factors, and inhibition of the ERK pathway blocks apoptosis. ERK activation also occurs in animal models of ischemia- and trauma-induced brain injury and cisplatin-induced renal injury, and inactivation of ERK reduces the extent of tissue damage. In some studies, ERK has been implicated in apoptotic events upstream of mitochondrial cytochrome c release, whereas other studies have suggested the converse: ERK acts downstream of mitochondrial events and upstream of caspase-3 activation. ERK also can contribute to cell death through the suppression of the anti-apoptotic signaling molecule, Akt. Here we summarize the evidence and mechanism of ERK-induced apoptosis in both cell culture and in animal models.

## General cascades of cell death

Cell death is divided into at least two categories, apoptosis and necrotic cell death. Apoptosis is a tightly orchestrated series of events that requires ATP and includes potassium efflux, cell shrinkage, mitochondria protein release and caspase activation (Yu et al., 2001; Boatright and Salvesen, 2003; Yu, 2003). In contrast, necrotic cell death is initiated by mitochondrial inhibition and the loss of ATP, and is associated with the breakdown of the cytoskeleton, cellular swelling, and progressive increases in plasma membrane permeability (Majno and Joris, 1995; Chen et al., 2001; Harriman et al., 2002; Liu et al., 2004). In general, apoptosis usually involves individual, noncontiguous cells whereas necrotic cell death usually involves multiple, contiguous cells (Columbano, 1995; Boatright and Salvesen, 2003). Interestingly, the extent of exposure to an insult determines the nature of cell death and both forms can occur simultaneously in tissue: apoptosis results from milder insults whereas necrosis follows more severe insults (Columbano, 1995) (Majno and Joris, 1995).

Extensive studies to elucidate the mechanisms by which apoptosis is induced and transduced have resulted in the generally accepted theory that intrinsic and extrinsic mechanisms are involved (Table 1) (Fadeel and Orrenius, 2005; Kim et al., 2006). Intrinsic pathways are activated by specific stress stimuli and lead to mitochondrial cytochrome c release, the activation of caspase-9 through Apaf-1, and the activation of executioner caspases (e.g. caspase-3) (Fadeel and Orrenius, 2005). The extrinsic apoptotic pathway is initiated by the activation of a death receptor, such as the TNF- $\alpha$  receptor and Fas, and caspase-8 (Kim et al., 2006). Activated caspase-8 can directly activate executioner caspases and/or induce cleavage of Bid to truncated Bid (tBid). tBid

is translocated to the mitochondria, prompting cytochrome c release and subsequent activation of caspase-3 (Li et al., 1998; Runden et al., 1998).

Concomitant with the activation of apoptotic pathways, survival signaling pathways are activated. Among them, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is activated by many apoptotic stimuli and plays a critical role in balancing apoptosis (Yang et al., 2004). Although the ERK pathway is attributed to survival for many cell types, ERK activation is now thought to contribute to apoptosis also.

### **ERK signaling pathway**

ERK belongs to a family of MAPKs, which is comprised of ERKs, c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), and p38 kinases. Within the ERK group there are eight isoforms (i.e. ERK1, 2, 3, 4, 5, 6, 7, and 8) (Bogoyevitch and Court, 2004; Yoon and Seger, 2006) of which ERK1/2 have been extensively studied. ERK1 and ERK2 are regulated by the dual-specificity kinases MEK1 and MEK2 through phosphorylation at both a threonine and an adjacent tyrosine residue within a dual-specificity motif (Thr-Glu-Tyr). MEK1 and MEK2 can be activated by multiple MAPK kinase kinases, although Raf kinase family members typically serve as dedicated MEK1/2 activators (Garrington and Johnson, 1999). Low molecular weight G-proteins (Ras, Rac, Rho, Cdc42, etc.), each associated with growth factor receptor activation and cellular adhesion can contribute to Raf activation either directly or indirectly. (Peyssonnaud and Eychene, 2001).

ERK1/2 also is activated in response to various stress stimuli through divergent mechanisms involving the Ras-Raf-MEK pathway (Strniskova et al., 2002; Kyosseva, 2004; Roux and Blenis, 2004; Baines and Molkentin, 2005; Rennefahrt et al., 2005).

Depending upon the cell type, the stimulus, and the duration of activation, a variety of biological responses (i.e. cell proliferation, migration, differentiation, and apoptosis) are associated with ERK activation (Strniskova et al., 2002; Kyosseva, 2004; Roux and Blenis, 2004; Baines and Molkenin, 2005; Rennefahrt et al., 2005). This pro-apoptotic perspective of ERK will comprise the remainder of this review.

### **ERK signaling mediates apoptosis in cultured cells**

Bhat *et al.*, first reported that inhibition of ERK using the MEK1 inhibitor PD98059 rescues oligodendrocytes from H<sub>2</sub>O<sub>2</sub>-induced cell death (Bhat and Zhang, 1999) and this observation was subsequently confirmed in Hela cells (Wang et al., 2000), cortical neurons (Lesuisse and Martin, 2002), and primary  $\beta$ -cells (Pavlovic et al., 2000) (Table 1). In renal cell lines (LLC-PK1; OK) and primary cultures of renal proximal tubular cells, inhibition of ERK improved cell survival by inhibiting apoptosis after cisplatin exposure (Ishikawa and Kitamura, 2000; Nowak, 2002; Kim et al., 2005). The pro-apoptotic role of ERK in renal epithelial cells is not limited to cisplatin exposure; ERK activation is also associated with cell death induced by reactive oxygen species (ROS) (Tikoo et al., 2001; Ramachandiran et al., 2002; Dong et al., 2004), *E. coli* toxins (Chen et al., 2004), zinc (Matsunaga et al., 2005), and cephaloridine (Kohda et al., 2003). ERK activation also has been implicated in cell death induced by deprivation of survival factors. For example, withdrawal of survival factors from mouse kidney proximal tubular epithelial cells led to a progressive increase in ERK1/2 activity, and inhibition of ERK1/2 maintained cell survival (Sinha et al., 2004). Finally, ERK1/2 activation is involved in doxorubicin-induced apoptosis in human hepatoma cell lines (HepG2 and Huh-7) (Alexia et al., 2004), amyloid-induced neurotoxicity in primary hippocampal neurons (Medina et

al., 2005) and CD40-mediated apoptosis in cholangiocytes (Ahmed-Choudhury et al., 2006).

Most of the above-mentioned studies addressed the role of ERK in apoptosis using MEK inhibitors PD98059 or U0126 (Alessi et al., 1995; Favata et al., 1998). Recently, Kim *et al.* used molecular approaches to examine the role of the ERK pathway in cell death of renal epithelial cells (Kim et al., 2005). They found that transient transfection of constitutively active MEK1 increased H<sub>2</sub>O<sub>2</sub>-induced apoptosis whereas the dominant-negative mutant of MEK1 led to an inhibition of H<sub>2</sub>O<sub>2</sub>-mediated cell death. Similarly, Goillot *et al.* demonstrated that interference with the ERK pathway by expression of dominant-negative MEK1 resulted in inhibition of Fas-mediated apoptosis (Goillot et al., 1997). These *in vitro* studies support a significant role for ERK in mediating apoptosis.

### **ERK signaling mediates injury-induced tissue damage in vivo**

ERK-mediated cell death also has been reported in numerous animal models. In a mouse model of stroke induced by transient occlusion of the middle cerebral artery (MCA) an increase in phosphorylated ERK in the nuclei of cortical cells was detected and pretreatment with PD98059 blocked ERK phosphorylation, resulting in a 55% decrease in focal infarct volume at 22 hr and a 36% decrease at 72 hr (Alessandrini et al., 1999). Using the same model, researchers confirmed these results using two additional MEK1 inhibitors, U0126 and SL327 (Namura et al., 2001; Wang et al., 2003; Wang et al., 2004b). In a gerbil model of forebrain ischemia induced by bilateral carotid artery occlusion, Namura *et al.* obtained similar results with MEK1 inhibitors (Namura et al., 2001). ERK also was rapidly activated in damaged tissue in a controlled cortical-impact

model of traumatic injury in mice, and ERK pathway inhibition with PD98059 prior to trauma resulted in a significant reduction of cortical lesion volumes 7 days after trauma (Mori et al., 2002; Clausen et al., 2004), Confocal immunohistochemistry showed that phospho-ERK co-localized with the neuronal nuclei marker Neu-N in the injured brain.

However, increased phospho-ERK1/2 staining was also detected in neurons that survived ischemic injury in a rat and mouse model of focal cerebral ischemia (Irving et al., 2000; Namura et al., 2001) suggesting that ERK1/2 activation may mediate cell survival in these areas. In support of an anti-apoptotic role of ERK, Gonzalez-Zulueta *et al.*, reported that activation of the Ras/ERK cascade was critical for the development of ischemic tolerance in cortical neurons (Gonzalez-Zulueta et al., 2000). Finally, inhibition of ERK1/2 with PD98059 failed to protect gerbils against ischemic cell death in the hippocampal CA1 region (Sugino et al., 2000) and did not block cerebral endothelial cell death after hypoxia-reoxygenation (Lee and Lo, 2003). These differences in the outcome of ERK activation may be attributed to the differences in the experimental models.

In a mouse model of cisplatin-induced nephrotoxicity, Jo *et al.*, observed that ERK1/2 phosphorylation increased mainly in distal tubules and collecting ducts 24 hr after cisplatin administration and persisted until 72 hr (Jo et al., 2005). Inhibition of ERK phosphorylation by U0126 pretreatment reduced tissue damage and improved renal function. A crucial role of ERK activation in mediating renal cell injury also was observed in rat renal cortical slices treated with cephaloridine, a cephalosporin antibiotic (Kohda et al., 2003). Further, serum thymic factor attenuated cisplatin nephrotoxicity by suppressing cisplatin-induced ERK activation (Kohda et al., 2005). These studies, coupled with the *in vitro* studies, reveal that ERK is a critical mediator in nephrotoxicity.



Although ERK is activated after renal ischemia/reperfusion injury in animals (Park et al., 2001), the role of ERK in ischemia/reperfusion-induced renal injury remains to be established.

### **Mechanisms of ERK-induced apoptosis**

#### *ERK-mediated regulation of the intrinsic pathway*

Because the intrinsic pathway is characterized by mitochondrial outer membrane permeabilization, cytochrome c release, and caspase activation, the association of ERK with these events has been investigated *in vivo* and *in vitro*. *In vivo* studies revealed that ERK1/2 acts upstream of caspase-3 in cisplatin-induced cell death in the kidney (Jo et al., 2005) and in the brain after ischemia/reperfusion (Wang et al., 2003). Using HeLa cells, Wang *et al.* showed that ERK1/2 inhibition blocks cytochrome c release and subsequent activation of caspase-3 in cisplatin-induced apoptosis (Wang et al., 2000). In a renal cell line (OK cells), Kim *et al.* reported that ERK activation is required for mitochondrial membrane depolarization, cytochrome c release, and caspase-3 activation after cisplatin exposure (Kim et al., 2005). Nowak reported that phosphorylated ERK1/2 was detected in mitochondria and associated with loss of mitochondrial function in cisplatin-treated primary cultures of renal proximal tubular cells (Nowak, 2002). However, ERK inhibition blocked caspase-3 activation without affecting cytochrome c release from mitochondria (Nowak, 2002). Thus, ERK1/2 may act on mitochondria to cause cytochrome c release and/or may regulate activation of caspase-3 downstream of cytochrome c release.

ERK may act on mitochondria through Bax and/or p53. Bax is a member of the Bcl-2 family of proteins and acts as pro-apoptotic molecule. During apoptosis induced

by a variety of stimuli, Bax is translocated to the mitochondria, where it promotes the release of pro-apoptotic proteins from the intermembrane space (Degli Esposti and Dive, 2003). In renal epithelial and osteoblastic cells, Bax expression is increased after cisplatin or H<sub>2</sub>O<sub>2</sub>, and inhibition of the ERK pathway decreased Bax expression (Kim et al., 2005; Park et al., 2005). Similar to the role of ERK in the regulation of Bax expression, ERK activation is also associated with up-regulation of p53 expression in HeLa cells treated with shikonin (a red naphthoquinone pigment isolated from the ground rhizome of lithospermum erythrorhizon ) (Wu et al., 2005) and in lens epithelial cells treated with calcimycin, a calcium mobilizer (Li et al., 2005). In the presence of apoptotic stimuli, p53 has been reported to translocate to the mitochondria to inhibit the action of the anti-apoptotic Bclx through the formation of a p53/Bclx complex (Petros et al., 2004) or to directly promote the pro-apoptotic activities of Bak (Mihara et al., 2003; Leu et al., 2004). Inhibition of Bclx and induction of Bax activity leads to cytochrome c release and caspase activation (Moll et al., 2005). In addition, ERK may regulate apoptosis via direct phosphorylation of p53. ERK can induce p53 phosphorylation at serine residue 15 (Persons et al., 2000) and inactivation of ERK resulted in p53 dephosphorylation and inhibited apoptosis (Brown and Benchimol, 2006). Together, these data suggest that Bax and p53 are important components in the ERK mediated apoptotic signaling pathways.

#### *ERK-mediated regulation of the extrinsic pathway*

ERK may induce apoptosis through regulation of the extrinsic apoptotic pathway. Jo *et al.* examined the effect of ERK inhibition on TNF- $\alpha$  expression and subsequent caspase-3 activation in cisplatin-induced acute renal failure in mice (Jo et al., 2005). They found that inhibiting ERK1/2 reduced TNF- $\alpha$  expression, caspase-3 activation, and

apoptosis in kidney tissue, suggesting that ERK1/2 pathway may also participate in apoptosis by increasing an upstream signal for TNF- $\alpha$  production. It is established that increases in interleukin IL- $\beta$  are closely associated with brain injury after cerebral ischemia (Barone and Feuerstein, 1999; Emsley and Tyrrell, 2002). Recently, Wang *et al.* showed that cerebral ischemia in mice induces ERK activation and interleukin-1 $\beta$  (IL-1 $\beta$ ) expression, and inhibition of ERK activation by U0126 prevented increases in IL-1 $\beta$  mRNA (Wang et al., 2004a). These studies implicate ERK-mediated expression of death ligands and pro-inflammatory cytokines as an important mechanism in exacerbating tissue injury.

The extrinsic apoptotic pathway relies on the activation of an initial caspase, caspase-8. Cagnol *et al.* examined the effect of ERK pathway activation on caspase-8 and apoptosis in HEK 293 cells that express an inducible form of Raf-1 (Raf-1:ER)(Cagnol et al., 2006). They showed that prolonged ERK stimulation activated caspase-8 and potentiated Fas signaling and apoptosis whereas expression of Bcl-X<sub>L</sub>, an inhibitor of apoptosis, did not significantly alter the apoptotic rate (Cagnol et al., 2006). These data suggest that ERK can regulate the apoptotic pathway at the level of caspase-8.

#### *ERK-mediated suppression of survival signaling*

Promotion of cell death by ERK activation also may result from the suppression of survival signaling pathways. The phosphoinositide-3-kinase (PI3K)-Akt pathway plays a critical role in the regulation of cell survival and most growth and survival factors activate this pathway (Amaravadi and Thompson, 2005). Recently, it was reported that withdrawal of soluble survival factors from primary cultures of mouse renal proximal tubular cells led to ERK1/2 activation that was accompanied by a gradual decrease in Akt

activity and apoptosis (Sinha et al., 2004). Inhibition of ERK1/2 with U0126 or PD98059 in the cells deprived of survival factors not only prevented the decline in Akt activity, but also resulted in cell survival (Sinha et al., 2004). These results support the idea that ERK1/2 activation in response to survival factor deprivation contributed to cell death via the suppression of the Akt pathway. Although the precise mechanism by which ERK1/2 inhibits Akt remains unclear, ERK1/2 and Akt have been reported to coexist in a multimolecular complex containing at least ERK1/2, Akt, ribosomal S6 kinase 1 (RSK), and phosphoinositide-dependent kinase 1 (PDK1) (Sinha et al., 2004). Whether ERK suppresses Akt activity through regulation of RSK and PDK1 or through other signaling molecules requires further investigation.

#### *Upstream inducers of ERK in apoptosis*

ERK activation can be induced by different stimuli via distinct mechanisms. For example, Arany *et al.* reported that the EGF receptor mediates ERK activation, and inhibition of the EGF receptor blocked cisplatin-induced ERK activation and apoptosis in mouse renal proximal tubules (Arany et al., 2004). Similarly, Lee *et al.* showed that H<sub>2</sub>O<sub>2</sub> triggers EGFR activation, and EGF receptor inactivation attenuated apoptosis in OK cells (Lee et al., 2005). Consequently, the death signal may initiate at a cellular membrane receptor and then propagate through an ERK-mediated signaling pathway.

Ras and Raf are downstream of growth factor receptors and mediate activation of MEK-ERK (Magnuson et al., 1994; Downward, 1998). Li *et al.* demonstrated that calcimycin-induced apoptosis of lens epithelial cells is mediated by MEK and ERK through a Ras and Raf-dependent pathway (Li et al., 2005). Woessmann *et al.*, showed that Ras-mediated activation of ERK by cisplatin induces cell death in osteosarcoma and

neuroblastoma cell lines (Woessmann et al., 2002). In addition, calcium can induce ERK and apoptosis through a Ras-dependent and Raf-independent mechanism (Vossler et al., 1997; Li et al., 2005). Src also can transduce EGF receptor activation to ERK in cisplatin-induced apoptosis of mouse proximal tubular cells (Arany et al., 2004). In contrast, Sinha *et al.* reported that growth factor depletion-mediated cell death is dependent on ERK but not Raf (Sinha et al., 2004). These data suggest that divergent intermediates are involved in transducing the death signal from the cellular membrane to ERK.

ROS play an important role in regulating cellular events leading to ERK activation and subsequent apoptosis. In addition to exogenous ROS activation of ERK and apoptosis, cellular production of ROS—due to a stimulus—can result in ERK activation. For example, cisplatin-induced cytotoxicity is closely related to increased generation of ROS (Sasada et al., 1996; Miyajima et al., 1997). ERK activation prompted by asbestos or the deprivation of growth factors can be blocked by the addition of ROS scavengers. Furthermore, H<sub>2</sub>O<sub>2</sub> generation and ERK activation are involved in 2,3,5-tris(glutathion-S-yl)hydroquinone (TGHQ)-induced cell death in LLC-PK1 cells (Tikoo et al., 2001; Ramachandiran et al., 2002; Dong et al., 2004). These studies suggest that ROS produce a specific environment that results in ERK-induced cell death.

### **The role of ERK5 in apoptosis**

Studies examining the role of ERK in cell death have primarily relied upon MEK1 inhibitors, U0126 and PD98059. Recently, it was reported that biological actions previously attributed to ERK1/2 may, in fact, be mediated by ERK5, a newer member of the MAPK family, because PD98059 and U0126 inhibit MEK1/2 as well as MEK5

(Kamakura et al., 1999; Cavanaugh et al., 2001). ERK5, also called big MAP kinase 1 (BMK1), contains a dual-phosphorylation motif (Thr-Glu-Tyr) similar to that in ERK1/2 and the N-terminal half of ERK5 shares sequence homology with other members of the MAPK family. However, a large C terminus and a unique loop-12 sequence distinguish it from ERK1/2. ERK5 is phosphorylated and activated by MEK5, but not by MEK1 or MEK2 (English et al., 1995; Zhou et al., 1995).

Similar to ERK1/2, activation of ERK5 plays a role in cell survival in response to a variety of stimuli, including oxidants and toxicants (Suzaki et al., 2002; Pi et al., 2004). Recently, ERK5 activation has been shown to promote apoptosis. For example, Sturla *et al.* reported that over-expression of ERK5 increased apoptosis in two medulloblastoma cell lines and primary cultures of patched heterozygous mouse medulloblastomas upon exposure to neurotrophin-3 whereas expression of small interfering RNA for the ERK 5 activator, MEK5, inhibited neurotrophin-3-induced apoptosis (Sturla et al., 2005). Furthermore, inhibition of myocyte enhancer factor (MEF2), a specific target of ERK5, by a dominant-negative mutant of MEF2 blocked MEK5/ERK5-induced cell death (Sturla et al., 2005). Although ERK5 has an apparent role in cell death, the mechanisms for this are unclear. Because many stimuli that induce ERK1/2 activation also activate ERK5, it is tempting to speculate that the ERK5 pathway may also be involved in the death of other cell types.

### **Conclusions and future directions**

Although ERK has generally been considered a survival signaling pathway, clear evidence exists that the ERK pathway mediates apoptosis induced by different stimuli in different tissues. The mechanisms by which ERK mediates apoptosis remain poorly

understood, and may occur at different levels. ERK1/2 may act upstream of mitochondrial cytochrome c release and caspase-3 activation through up-regulation of Bax and p53 and through suppression of Akt-mediated survival signaling. Further studies are necessary to elucidate the activated signal transduction upstream and downstream of the cascades and to define the crosstalk among the cascades and other signaling pathways.

The rationale for a signaling pathway being responsible for cell survival and apoptosis is not clear. At present, the signaling of survival or apoptosis by ERK appears to be dependent on the model system and injury paradigm. Furthermore, the kinetics and duration of ERK activation may play an important role in influencing its effect on cell fate. It has been reported that prolonged ERK activation is accompanied by the pro-apoptotic effect of ERK (di Mari et al., 1999) whereas a transient activation of ERK protects cells from death (Arany et al., 2004). Nevertheless, the protective effect of ERK inhibition has been reported in animal models of ischemia/reperfusion, and ERK activation was reported in humans who had experienced a stroke (Slevin et al., 2000), so the protection strategies which target these mechanisms merit further exploration.

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## Figure Legends

**Figure 1. Mechanisms of ERK-mediated apoptosis.** ERK may regulate apoptosis and cell survival at multiple points that include increasing p53 and BAX action, increasing caspase-3 and caspase-8 activities, decreasing Akt activity, and increasing TNF- $\alpha$  production.

**Table 1. Examples of ERK mediated apoptosis in different cell types and stimuli**

<b>Cell types</b>	<b>Inducers</b>	<b>References</b>
Oligodendrocyte	H <sub>2</sub> O <sub>2</sub>	(Bhat and Zhang, 1999)
Hippocampal neurons	Amlloid	(Medina et al., 2005)
Glial cells	gluthathione depetion	(de Bernardo et al., 2004)
CGN (cerebellar granule neurons)	low potassium	(Subramaniam et al., 2003; Subramaniam et al., 2004)
	steroid hormone	(Wong et al., 2003)
Pyramidal cells	Okadaic acid	(Runden et al., 1998)
Renal proximal tubular cells	Cisplatin	(Nowak, 2002; Arany et al., 2004; Kim et al., 2005)
Hepatoma cell line (HepG2)	doxorubicin	(Alexia et al., 2004)
HeLa (cervical carcinoma cells)	Cisplatin	(Wang et al., 2000)
Renal epithelial cells	Growth factors depletion	(Sinha et al., 2004)
Renal epithelial cells	Escherichia coli toxin	(Chen et al., 2004)
Renal epithelial cells	ROS	(Tikoo et al., 2001; Ramachandiran et al., 2002; Dong et al., 2004)
Osteoblastic cells	H <sub>2</sub> O <sub>2</sub>	(Park et al., 2005)
Pancreatic beta-cells	interleukin-1 beta	(Pavlovic et al., 2000)
TM4 cells (Sertoli cell line)	CD95L	(Ulisse et al., 2000)
Lens epithelial cells	Calcium	(Li et al., 2005)
Bone marrow stromal cells	Leptin	(Kim et al., 2003)
B65 cell line (dompamine neuron)	6-hydroxydopamine	(Kulich and Chu, 2001)
SH-SY5Y (neuroblastoma cell line)	1-Methyl-4-phenylpyridinium	(Gomez-Santos et al., 2002)

Figure 1

