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Original Article

Midkine inhibits apoptosis via extracellular signal regulated kinase (ERK) activation in PC12 cells

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Midkine (MK) is a new member of the family of heparin-binding neurotrophic factors. MK has several important biological effects and plays an important role in the development and survival of neurons. The mechanism by which MK exerts its neurotrophic actions, however has not been sufficiently clarified. To understand the intracellular pathway activated by MK, we established an apoptosis-induction system with the neuronal cell line PC12 and studied the involvement of the mitogen-activated protein kinase (MAPK) cascade in neuroprotective actions of MK. We demonstrate here that MK rescued PC12 cells from apoptosis induced by serum deprivation in a dose-dependent manner. MK also activated extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), which are well known as signal transducer acting downstream several receptors. PD98059, an inhibitor of MAPK kinase (MAPKK), inhibited ERK activation and also prevented the trophic effect of MK. These results indicate that MK exerts its neuroprotective actions mainly via ERK activation.

Key words: midkine; apoptosis; PC12; MAPK; ERK

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1. Introduction

Midkine (MK) was discovered by analysis of mRNA in retinoic-acid-treated embryonal carcinoma cells[1]. It has been reported to be a member of the heparin-binding neurotrophic factor family, and is a polypeptide rich in basic amino acids and cysteine with a molecular mass of 13 KDa[1,2].

MK gene expression is found in the brain surface in 13-day mouse embryos, and in remodeling mesodermal tissues during embryogenesis[3,4,5]. MK expression has also been found in various tumors such as ovarian cancer, breast cancer, Wilms' tumor, and astrocytoma[6,7,8,9].

Moreover, MK has been shown to have neurotrophic activity on neurons isolated from the embryonal murine spinal cord, dorsal root ganglion, mesencephalon, and human brain[10,11,12]. Although MK exerts neurotrophic effects on various neurons, the precise intracellular mechanisms which are activated by MK have not been clarified.

The mitogen-activated protein kinase (MAPK) signal transduction pathway, including the extracellular signal-regulated kinase (ERK), is important for transmitting stimulation by neurotrophic factors[13]. Although the specific receptor for MK has not yet been cloned, a previous study revealed that MK induces tumor proliferation via a high-affinity receptor located on the cell surface, which was associated with the janus non-receptor tyrosine kinases JAK1 and JAK2[14]. Therefore, it would seem reasonable to examine whether the neurotrophic actions of MK are regulated

via the MAPK signaling pathway.

In this study, we have shown that MK prevents apoptosis in a dose-dependent manner induced by serum deprivation in PC12 cells, a cell line established from rat adrenal pheochromocytoma. We also found that administration of MK provokes the activation of ERKs, and that inhibition of ERKs decreases the trophic effects of MK. These findings suggest that MK exerts trophic effects on PC12 cells via ERK activation and thus inhibits apoptosis induced by serum deprivation.

Materials and Methods

Cell culture

PC12 rat pheochromocytoma cells were purchased from Riken cell bank (Tokyo, Japan, RCB0009). The cells were maintained in Dulbecco's minimum essential medium (DMEM) (Gibco Life Technologies, N.Y., NY, USA) with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenaxa, KS, USA, lot no. 7B4003) at 37℃ in 5% CO_2. For replating, PC12 cells were incubated for 30 min in Phosphate-buffered saline (PBS) (Takara, Otsu, Japan) containing 0.25% trypsin, and then the same volume of DMEM containing 10% FBS was added to stop the enzymatic reaction. The cells were dissociated into single cells by gently pipetting. Then the cells were plated onto poly-D-lysine (MW 96000) (Sigma Chemical Co., MO, USA) coated glass coverslips or 96-well plate at a density of 1×10^5 cells /cm^2. The cells were cultured overnight in DMEM with 10% FBS, and then the cells were washed in minimum essential medium (MEM) (Gibco Life technologies, N.Y., NY, USA) 3 times and were cultured in MEM alone or with reagents tested.

Purification of recombinant MK

Recombinant MK secreted by L cells, which were transfected with MK cDNA, was purified from the supernatant of L cell culture medium by chromatography on phenyl-Sepharose gel and heparin-Sepharose gel as described previously[3].

Detection of apoptotic cells

Apoptotic cells were detected by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method, with terminal transferase and biotin-16-2′ deoxyuridine 5′ triphosphate according to the manufacture's protocol (Boeringer Mannheim, Mannheim, Germany). To visualize the nick-end labeled products, the cultures were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for several minutes. The number of TUNEL-positive apoptotic cells and TUNEL negative cells were counted on photomicrographs. Quadruplicate experiments were performed under the same conditions. Statistical significance was assessed by Student's t-test.

MTT assay

The MTT assay is based upon the change of 3-(4,5-dimethyl-thazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako, Tokyo, Japan), (a yellow tetrazolium salt) to insoluble purple formazan[15]. PC12 cells in 96-well plates were incubated for 24 hr in medium containing 10% FBS, and then the medium was changed to serum-free MEM containing various reagents. After 24 hr the medium was completely changed to MEM containing 500 μg/ml MTT solution. After 60 min of incubation in a water jacket incubator, the yellow MTT salt was changed to purple formazan, which was tightly attached to the bottom of the wells. To dissolve the formazan, the medium was changed to 100% Dimethylsulfoxide (DMSO) (Spectosol, Dojindo, Kumamoto, Japan), and then the plates were covered with plastic wrap and placed into an ultrasonic water bath for 10 min. Then the OD was measured at a wavelength of 570 nm. Each set of conditions was tested on the cells at least in quadruplicate, and the results were expressed as the mean and standard deviation. Statistical significance was assessed by Student's t-test.

Western blot analysis

Cells were harvested into sampling buffer (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 μM orthovanadium, 1.5 μM pepstatin, 2 μM leupeptin, and 0.7 μM aprotinin) on ice. The collected samples were homogenized, and the cell lysates were removed by centrifugation at 10,000$\times g$ for 20 min at 4℃. After the protein concentration was determined by a bovine serum albumin (BSA) protein assay kit (Pierce, Rochford, England), 12.5% SDS polyacrylamide gel electrophoresis was performed with 50 μg of total protein per sample. The separated proteins were transferred onto a nitrocellulose membrane, blocked with 4% BSA in Tris-buffered 0.2% Tween 20 (TBS-T) at 4℃ for 1 hr and incubated overnight at 4 ℃ with an anti-activated MAPK polyclonal antibody (Anti-active MAPK antibody, Promega, WI, USA) (1:1000 dilution). The membranes were washed three times with TBS-T, incubated with alkaline phosphatase-conjugated anti-rabbit IgG

(1:20000 dilution) for 3 hr at room temperature, washed twice in TBS-T, and then developed with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolephosphate p-toluidine salt (BCIP). To confirm the equal amount of samples were transferred to the membrane, the blot was re-probed with anti-pan MAPK polyclonal antibody (Anti-ERK 1 antibody, Promega, WI, USA) (1:1000 dilution) which recognized both activated and non-activated ERKs for 5 hr. After the membrane was washed twice in TBS-T, peroxidase-conjugated anti-goat IgG (1:15000 dilution) secondary antibody was applied for 1 hr. After the membrane was washed twice in TBS-T, it was developed by the ECL system (Amersham, CEA, Sweden).

Results

MK suppresses apoptosis induced by serum free conditions

After incubation for 24 hr under serum-deprived conditions, the cultures were fixed with 4% paraformaldehyde for 120 min, and TUNEL staining was performed. Because TUNEL-positive cells were visualized by DAB, the DNA of apoptotic cells was stained a dark brown color in the nucleus. The number of TUNEL-positive cells was counted in triplicate independent experiments. Numerous TUNEL-positive cells were observed under serum free conditions (arrows, Figure 1b), and the addition of MK suppressed the increase in the number of TUNEL-positive cells. TUNEL positivity was observed in 60.4% of cells in serum free control cultures (Figure 2, column 2). In contrast, the addition of 100 ng/ml MK markedly suppressed the proportion of apoptotic TUNEL-positive cells to 27.3% (Figure 2, column 1, $p < 0.001$). These results show that serum deprivation induced apoptosis of PC12 cells in this system, and that MK rescued the cells from apoptosis.

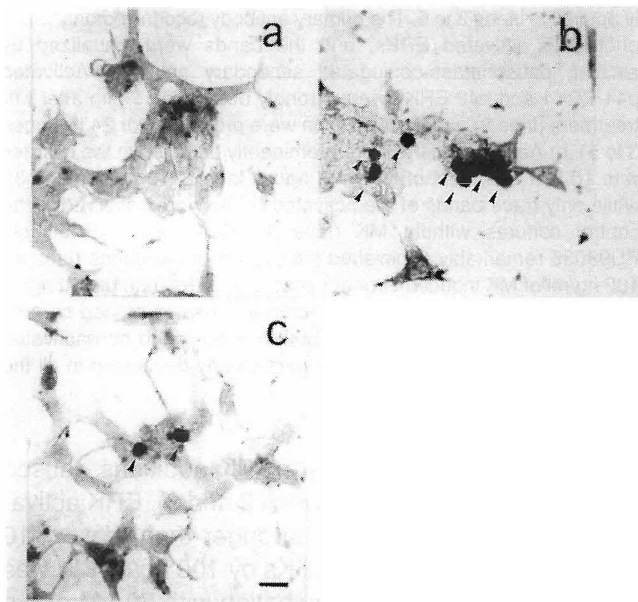

Figure 1. MK inhibits apoptosis of PC12 cells induced by serum-deprivation.

PC12 cells were incubated for 24 hr in DMEM with 10% FBS, and the medium was changed to serum-free medium 30 min before MK was added. Also 50 μM of the MAPKK inhibitor PD98059 was added 15 min before MK addition. PC12 cells were treated with serum-free conditions for 24 hr. Although numerous dark brown TUNEL-positive cells (arrows) were observed under the control conditions without MK (b), TUNEL-positive cells were markedly fewer than those in the control slides for the cultures treated with 100 ng/ml MK (a). Preincubation and coincubation with PD98059 increased TUNEL-positive cells in cultures with MK (c). Bar indicates 10 μm.

MK has a cytoprotective effect in a dose-dependent manner

The relationship between cell viability and MK concentration is shown in Figure 3. After PC12 cells were cultured in DMEM containing 10% FBS for 24 hr in 96-well plates, the medium was changed to MEM alone. After 24 hr under serum-free conditions, the MTT

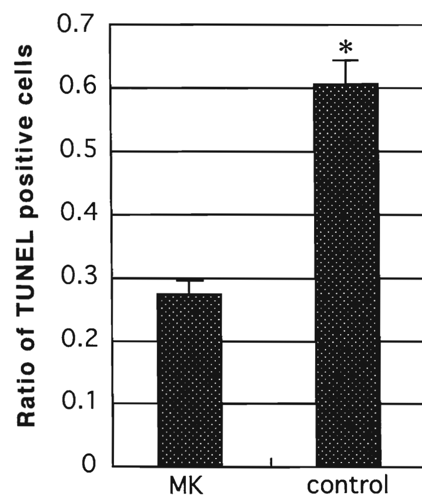

Figure 2. The ratio of TUNEL-positive cells was calculated under various conditions.

PC12 cells were incubated for 24 hr on coverslips in DMEM with 10% FBS, and the medium was changed to serum free medium with or without MK (100 ng/ml). After the coverslips were fixed with 4% PFA, they were processed for TUNEL staining. The slides were photographed, and then the cells were counted to analyze the ratio of TUNEL-positive cells in independent quadruplicate experiments. Twenty-four hrs after serum-deprivation, 60.4% of cells were TUNEL-positive in cultures without MK and 27.3% were TUNEL-positive with MK ($p < 0.001$ compared with control).

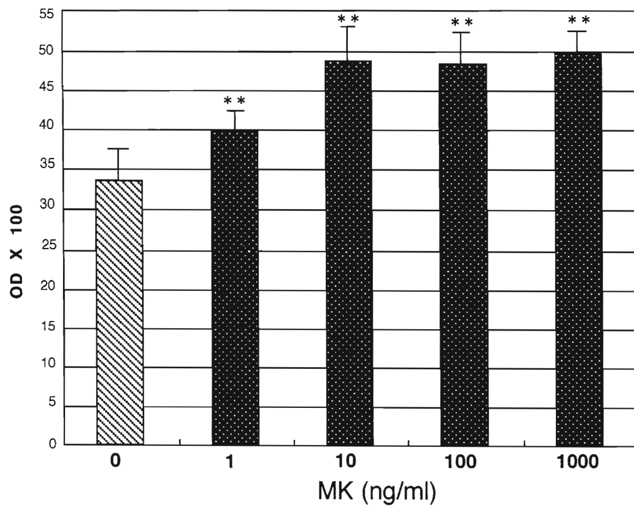

Figure 3. Dose-dependence of the effect of MK on cell viability.

PC12 cells were incubated for 24 hr in DMEM with 10% FBS, and the medium was changed to serum-free medium 30 min before MK was added. PC12 cells were incubated with MK at concentrations of 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml and 0 ng/ml (control condition) for 24 hr, and the number of viable cells was determined by the MTT assay. MK increased the number of viable cells in a dose-dependent manner (columns 2 to 4, $p < 0.001$ compared with control), and an increase of OD was observed with the addition of as low as 10 ng/ml of MK (columns 3 to 5).

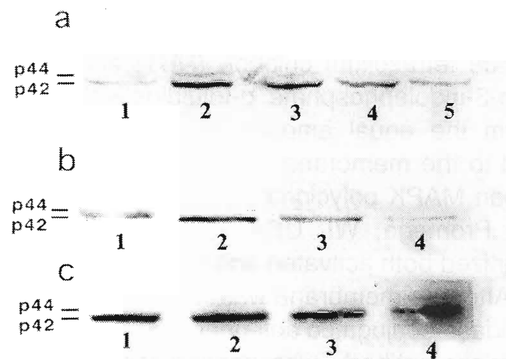

Figure 4. Activation of ERKs by MK was in a time- and a dose-dependent manner. Activation of ERKs by MK was reduced in the presence of PD98059.

PC12 cells were incubated for 24 hr in DMEM with 10% FBS, and the medium was changed to serum-free medium 30 min before MK was added. To examine whether 50 μM PD98059 blocked the activation of ERKs, the cells were preincubated with 50 μM PD98059 for 15 min, and then treated with 100 ng/ml of MK for another 15 min. Cell lysates were collected into buffer containing several enzyme inhibitors on ice. After centrifugation, 50 μg of total cellular protein was used for western blotting. a) The sample collected before MK treatment was applied in lane 1. The samples collected 15 min, 30 min, 12 hr, and 24 hr after 100 ng/ml MK treatment were respectively applied in lanes 2 to 5. The primary antibody recognized only phospholylated activated ERKs, and the bands were visualized by alkaline phosphatase-conjugated secondary antibody. Activated p44 ERK1 and p42 ERK2 were strongly observed 15 min after MK treatment (lane 2) and the activation were prolonged for 24 hr (lanes 3 to 5). b) Activated ERKs were prominently detected in the cultures with 15 min treatment of MK (100 ng/ml, lane 2; 10 ng/ml, lane 3), while only trace bands of the activated ERKs were observed in the control cultures without MK (lane 1). Coexistence of 50 μM PD98059 remarkably diminished the density of the bands (lane 4). 100 ng/ml of MK induced stronger activated ERKs than did 10 ng/ml of MK (lanes 2, 3). c) The same membrane was re-probed by anti-ERK antibody, which recognized both activated and non-activated ERKs. The bands of p42 and p44 were clearly developed in all the lanes.

assay was performed. Because viable cells converted soluble yellow tetrazolium salt MTT to an insoluble purple formazan product, the amount of insoluble formazan reflected cell viability. To quantify the amount of formazan, we used an ELISA reader to measure the OD at 570 nm. When cells were cultured under serum-free control conditions, the OD was 0.334 (Figure 3, column 1), whereas the addition of 1000 ng/ml MK increased the OD to 0.499 (Figure 3, column 5, $p < 0.001$ compared with control). The increase in absorbance was observed by the addition of as low as 10 ng/ml of MK (Figure 3, columns 2–5).

MK activates MAPKs (ERK1 and ERK2)

a) Cells cultured with or without MK were collected in the buffer, and then activated p44 ERK1 and p42 ERK2 in the cell lysates were detected by Western blotting. At first, the time course of ERKs activation by MK was analyzed. ERKs were observed 15 min after MK was added (Figure 4a, lane 1), and the activation was prolonged for 24hr. b) To analyze the relationship between MK concentration and ERKs activation, we applied 100 ng/ml and 10 ng/ml MK and the samples were collected after a 15min incubation period. There were only trace of activated ERKs in the cell lysates under serum-free control conditions (Figure 4b, lane 1), while 100 ng/ml and 10 ng/ml MK additions caused ERK activation (Figure 4b, lanes 2 and 3). ERK activation by 100 ng/ml MK was stronger than that by 10 ng/ml of MK. Activation of ERKs by 100 ng/ml MK was blocked by a 15 min pre-incubation with 50 μM of the MAPKK inhibitor PD98059, and the bands of activated ERKs were remarkably diminished (Figure 4b, lane 4). c) To confirm that the proteins were adequately transferred onto the membrane, the same membrane was re-probed with anti-ERK 1 antibody, which recognized both the activated and inactivated forms of ERKs. Almost the same band density was found in all the lanes (Figure 4c, lanes 1-4). Thus, MK significantly activated ERKs in a time and dose dependent manner, and 50 μM PD98059 strongly diminished ERK activation by

MK.

The cytoprotective effects of MK were diminished by PD98059 treatment

The MTT assay and TUNEL staining were performed to analyze whether MAPK activation was related to the cytoprotective effect of MK. The number of viable cells in control cultures was lower compared with that in cultures following MK treatment (Figure 5a, column 1, $p < 0.001$) in the MTT assay, and the number of TUNEL-positive cells was higher (Figure 5b, column 1, $p < 0.001$). The number of viable cells in cultures containing MK was 1.9-fold greater than that in control cultures. When cultures were preincubated with 50 μM PD98059 for 15 min and then treated with MK, the number of viable cells was 67.6% of that in cultures treated with MK alone (Figure 5a, column 2, $p<0.001$) in the MTT assay. In addition to this, the number of TUNEL-positive cells in the cultures treated with PD98059 and MK (Figure 1c) was 1.7-fold higher than that in cultures containing MK alone. These findings support the suggestion that MK exerts its cytoprotective effects via ERK activation.

Discussion

We obtained the following results on the trophic effects of MK using PC12 cells cultured under serum-deprived conditions: (a) MK suppressed apoptosis induced by serum deprivation; (b) MK exerted its cytoprotective effects in a dose dependent-manner; (c) MK activated ERKs, and a selective MAPKK inhibitor which inhibits the activation of ERKs inhibits the cytoprotective effects of MK. Thus, it was suggested that MK exerts its cytoprotective effects mainly via ERK activation.

Although the cytoprotective effects of MK have been reported previously, the precise mechanisms of these effects have not been sufficiently clarified. PC12 cells were established from rat adrenal pheochromocytoma[16] and are widely used to examine the signal transduction system. Serum deprivation induces apoptosis of PC12 cells and nerve growth factor (NGF) prevents such apoptosis[13,17]. We showed here that MK had similar cytoprotective effects on PC12 cells as does NGF.

The p42 and p44 MAPKs are called extracellular signal-regulated kinases (ERKs) and are reported to be activated by various growth factors[18,19]. It is well known that MAPK activation plays an essential role in cell survival, growth, and differentiation[19,20,21]. Continuous selective activation of ERKs prevents apoptosis induced by withdrawal of NGF from PC12 cells[13], and NGF activates ERKs continuously[17,21]. These findings suggest that the trophic effects of MK on neurons[10,11,12], and the viability of PC12 cells may be regulated by ERK activity. We therefore investigated the

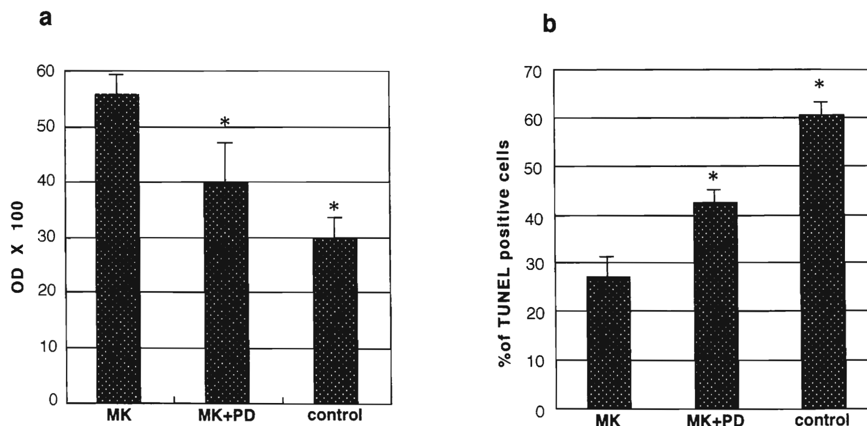

Figure 5. Concurrent treatment with PD98059 decreased the cytoprotective effect of MK.
PC12 cells were incubated for 24 hr in DMEM with 10% FBS, and the medium was changed to serum-free medium 30 min before MK was added. 50 μM PD98059 was added to culture medium 15 min prior to the addition of MK. a) Cultures with 100 ng/ml MK, 100 ng/ml MK together with 50 μM PD98059, and no reagent (serum-free control) were maintained for 24 hr. Then the MTT assay was performed, and the number of live cells was determined. In the presence of PD98059, MK did not exert the cytoprotective effects and the cell viability was decreased to 66% of that with MK alone (* $p < 0.001$ compared with MK alone. b) TUNEL staining was performed to confirm that MK has the cytoprotective effects and not proliferative effects on PC12 cells. Although 61.2% of the cells were TUNEL-positive under serum-deprived conditions , MK suppressed the ratio of TUNEL-positive cell number to 26.7%. Addition of PD98059 increased the ratio of TUNEL-positive cell number to 42.9% (* $p < 0.001$ compared with MK alone).

relationship between ERK activation and the neurotrophic effects of MK.

The compound 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) is a selective MAPKK inhibitor, and preincubation or coincubation with 50 μM PD98059 is reported to completely block the production of active form of ERKs[22]. Therefore, we added this compound 15 min before MK addition to block ERK activation. As a result, the cytoprotective effect of MK was diminished by PD98059. We confirmed that 50 μM PD98059 blocked the activation of ERKs by Western blot analysis (Figure 4b, lane 4). To examine whether PD98059 is cytotoxic for PC12 cells, we treated PC12 cells with PD98059 at various concentrations for 24 hr. We confirmed that 50 μM PD98059 did not show significant cytotoxity on the cells in the TUNEL assay (data not shown). These data strongly suggest that 50 μM PD098059 suppressed teh activation of ERKs triggerd by MK, which resulted in the diminition of the cytoprotective effects of MK.

Because the G401 cell line derived from rhabdoid kidney tumor expressed MK and anti-MK antibody partially inhibited the growth of the tumor, MK exerts the cell proliferative effect via an autocrine mechanism[3, 23]. In addition, MK specifically binds to high-affinity receptors on the cell surface of the G401 cells, and the receptors activate the JAK/STAT signal transduction pathway[23]. Thus it is strongly suggested that receptor-regulated signal transduction pathways are activated by MK in PC12 cells, although the MK receptors in PC12 cells have not yet been cloned.

The important role played by ERKs in trophic factor-mediated signalings in PC12 cells has been well documented[13,20], but the precise transduction pathway involved still remains controversial. Recently, the phosphatidylinositol-3′-kinase (PI3 kinase) pathway, in addition to the MAPK pathway, has been reported to be activated by NGF in PC12 cells[17]. In addition, insulin-like growth factor 1 (IGF-1) was shown to inhibit apoptosis via both the MAPK and PI3 kinase pathways[24]. Our present data in Figure 5 show that PD98059 only partially inhibited the survival-promoting actions of MK. One possibility that explains this result is that MK inhibits apoptosis via activation of other pathways in addition to the MAPK pathway, although we conclude that activation of ERKs plays a significant role in exerting the cytoprotective effects of MK.

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