Activated δ-opioid receptors inhibit hydrogen peroxide-induced apoptosis in liver cancer cells through the PKC/ERK signaling pathway

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Abstract. Apoptotic liver cancer cells have important roles in liver tumorigenesis and liver cancer progression. Recent studies have shown that δ-opioid receptors are highly expressed in human liver and liver cancer cells. The present study aimed to investigate the role of activated δ-opioid receptors on human liver cancer cell apoptosis and its interrelation with the mitochondria and the protein kinase C/extracellular-signal-regulated kinase (PKC/ERK) signaling pathway. H2O2 was used to induce apoptosis in human liver cancer cells. During apoptosis, mitochondrial transmembrane potentials were observed to decrease, cytochrome c expression was found to increase and B cell lymphoma 2 (Bcl-2) expression decreased. These findings suggested that H2O2-induced apoptosis was mediated through the mitochondrial pathway. Of note, activated δ-opioid receptors were observed to inhibit H2O2-induced apoptosis in human liver cancer cells. Following δ-opioid receptor activation, the number of apoptotic liver cancer cells decreased, mitochondrial transmembrane potentials were restored, cytoplasmic cytochrome c and Bcl-2-associated X protein expression decreased and Bcl-2 expression increased. These data suggested that δ-opioid receptor activation inhibited mitochondria-mediated apoptosis. In addition, activation of δ-opioid receptors was observed to increase the expression of PKC and ERK in human liver cancer cells. Furthermore, upon inhibition of the PKC/ERK signaling pathway, the protective effect associated with the δ-opioid receptor on liver cancer cell apoptosis was inhibited, which was not associated with the status of δ-opioid receptor activation. These findings suggested that the PKC/ERK signaling pathway has an important role in δ-opioid receptor-mediated inhibition of apoptosis in human liver cancer cells.

Introduction

Liver cancer cell apoptosis has an important role in the occurrence and development of liver cancer and is mediated through multiple pathways (1). Thus, the study of liver cancer cell apoptosis may have important clinical implications for the treatment of liver cancer and the maintenance of liver function. The δ-opioid receptor is a member of the opioid receptor family and is highly expressed in several human organs. Studies have shown that activated δ-opioid receptors stimulate the proliferation of myocardial cells in newborn rats (2) and have a protective role in ischemic-preconditioning of the heart and brain tissues (3,4). A previous study by our group demonstrated that activated δ-opioid receptors had a protective effect against apoptosis in liver cancer cells (5). These findings suggested that δ-opioid receptors have important roles in cell survival and proliferation. In addition to the central nervous system and the heart, δ-opioid receptors are highly expressed in liver and liver cancer cells (6,7). Furthermore, the δ-opioid receptor has been found to have a significant role in the occurrence and development of liver diseases, including hepatoma, viral hepatitis and hepatic cirrhosis (7-9).

H2O2 is commonly used to induce apoptosis (10). In the present study, different concentrations of H2O2 were added to cultured cell media for specific time periods. The mechanisms underlying reactive oxygen species-induced apoptosis include receptor activation, activation of the caspase cascade,
modulation of the expression of B-cell lymphoma (Bcl)-2 family member proteins and mitochondrial damage (11). The 
H₂O₂ model of apoptosis mimics the physiological conditions of hepatic ischemia and hypoxia. Apoptosis proceeds via two
major pathways: The death receptor pathway and the mitochon-
drial pathway (12). Mitochondrial apoptosis is initiated
through alterations in mitochondrial structure and function,
specifically by decreasing the mitochondrial transmembrane
potential. Large quantities of cytochrome c released from the
mitochondria activate the caspase cascade, resulting in the
activation of caspase-3 and apoptosis. A series of studies have
suggested that the δ-opioid receptor protects myocardial,
neuronal and liver cells through inhibiting the mitochondrial
apoptotic pathway (5,13,14). In the present study, 
H₂O₂ was found to induce human liver cancer cell apoptosis through
the mitochondrial pathway. Therefore, it was hypothesized that
activated δ-opioid receptors may regulate liver cancer
cell apoptosis through the mitochondrial pathway.

Protein kinase C (PKC) is a serine/threonine kinase, which
is widely expressed in human cells. In the unstimulated
state, PKC is distributed in an inactive form in the cytosol.
Following external stimulation, PKC is translocated from the
cytosol to the plasma membrane and is activated. The
PKC signaling pathway is involved in various biological
activities, in which it mediates proliferation and differentia-
tion in multiple cell types. Studies have shown that PKC has
a protective effect in ischemic-preconditioned livers (15).
Proliferation and apoptosis in normal liver and liver cancer
cells are closely associated with the PKC pathway (16-18). A
previous study by our group showed that activated δ-opioid
receptors and phosphorylated PKC participate in a common
signaling pathway (5).

Extracellular-signal-regulated kinase (ERK) was the
first mitogen-activated protein kinase (MAPK) to be identi-
fied and is the most studied MAPK member. ERK has two
isoforms, ERK1 and ERK2. The two phosphorylation sites,
a tyrosine residue and a threonine residue, are separated by
a glutamic acid residue, thus the phosphorylation motif of
ERK is TEY. p38 and c-Jun N-terminal kinase (JNK) are
stress-activated MAPKs. Studies have shown that the ERK
signaling pathway is involved in a wide range of biological
activities and induces cell growth, proliferation and
apoptosis (19). A growing body of evidence suggests that
G-protein-coupled receptors (GPCRs) activate ERK through
multiple mechanisms, including G-protein-dependent and
G-protein-independent pathways. It is well established
that ERK is a downstream effector of GPCR proteins, one
of which is the δ-opioid receptor. Studies have demonstra-
ted that δ-opioid receptors and ERK participate in the
same downstream signaling pathways (20,21). A study by
Xu et al (22) showed that δ-opioid receptors activate ERK
through G-protein- or arrestin-dependent mechanisms.
Therefore, in the present study it was hypothesized that
δ-opioid receptors may regulate apoptosis through activation
of the ERK pathway.

In the present study, 
H₂O₂ was used to induce apoptosis in cultured human liver cancer cells in vitro. The role of
δ-opioid receptors in the regulation of apoptosis and its inter-
relation with PKC, mitochondria and the ERK pathway was
then investigated in human liver cancer cells.

Materials and methods

Reagents. HepG2, HepH3B, SK-Hep-1 and LO2 cell lines
were obtained from the Cell Bank of The Chinese Academy
of Sciences (Shanghai, China). [d-Ala², d-Leu⁵] enkephalin
(DADLE), naltrindole, GF109203X, U0126, and MTT were
purchased from Sigma-Aldrich (St. Louis, MO, USA). The
5',6',6'-tetrachloro-1',3',3'-tetraethylbenzimidazolocarboxy-
cine iodide (JC-1) Mitochondrial Membrane-Potential Assay
kit was purchased from Abcam Plc (Cambridge, MA, USA).
RPMI-1640 and fetal bovine serum (FBS) were purchased from
Gibco-BRL (Carlsbad, CA, USA). An Annexin V-fluorescein
isothiocyanate (FITC) apoptosis kit was purchased from
Bio-Rad (Hercules, CA, USA). Phosphorylated PKC (rabbit,
monoclonal), Bcl-2 (rabbit, polyclonal), and Bcl-2-associated
X (Bax) antibodies (rabbit, polyclonal) were purchased from
Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).
The following antibodies were used for western blot analysis:
β-actin (sc-47778, diluted 1:1,000) and were obtained from
Santa Cruz Biotechnology, Inc. Cytochrome c and phos-
phorylated ERK antibodies were purchased from Abcam Plc.
The present study was approved by the Ethics Committee of
The Second Affiliated Hospital of Dalian Medical University
(Dalian, Liaoning, China).

Cell culture. Liver cancer cells were seeded at a density of
1x10⁶ cells/ml in T25 cell-culture flasks and cultured in
Dulbecco’s modified Eagle medium (Gibco-BRL) supple-
mented with 10% FBS, penicillin and streptomycin in an
incubator with 95% O₂ and 5% CO₂.

Experimental treatment. Liver cancer cells were cultured
for 12 h. Except for those in the control group, cells were
treated with various concentrations of 
H₂O₂ (10, 50, 100, 200 and 400 mM) for 12 h. While under 
H₂O₂ treatment, cells in the intervention groups were also treated with either the
δ-opioid-receptor agonist DADLE (0.01, 0.1, 1.0 or 10 µM),
the δ-opioid-receptor-specific inhibitor naltrindole, the PKC
inhibitor GF109203X (10 µM) or the ERK inhibitor U0126
(10 µM) for 12 h.

Cell viability assay. The MTT assay was used to analyze
cell viability. Human liver cancer cells were treated with
200 mM H₂O₂ and various concentrations of DADLE for 12 h
followed by incubation with 20 µl MTT solution [5 mg/ml in
phosphate-buffered saline (PBS), pH 7.4] for 4 h. The cell
media was then removed and the formazan crystals in each
well were fully dissolved in 200 µl dimethyl sulfoxide by
vortexing for 10 min. The absorbance value of each well was
measured and recorded using a microplate reader (FLx800™;
Bio-Tek Instruments, Inc., Winooski, VT, USA) at a wave-
length of 570 nm.

Detection of apoptosis using Annexin V/propidium iodide
(PI) double labeling. An Annexin V-FITC apoptosis kit was
used according to the manufacturer’s instructions. Cell death
was detected using flow cytometry (FACS Vantage SE flow
cytometer: BD Biosciences, Franklin Lakes, NJ, USA). Cells
positive for Annexin V and negative for PI were considered to
be early apoptotic cells. In brief, cells were harvested using
0.25% trypsin, washed with PBS three times, stained with 10 µl Annexin V and 5 µl PI and incubated in the dark at room temperature for 15 min. Cells were then analyzed using flow cytometry.

Detection of changes in mitochondrial membrane potential using JC-1 staining and flow cytometry. Cells were suspended at a concentration of 1x10^5 cells/ml, incubated with 10 µg/ml JC-1 staining solution, mixed thoroughly and incubated for 20 min. Non-conjugated JC-1 was removed using buffer, then cells were resuspended in buffer. Cells were analyzed using flow cytometry with an emission wavelength of 488 nm. The FL1-h and FL2-h values represent the intensities of red and green fluorescence, respectively. The results were quantitatively analyzed using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Isolation and purification of mitochondria. In each group, liver cancer cells were collected and suspended in pre-chilled extraction buffer [0.2 mol/l mannitol, 50 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l ethylene glycol tetraacetic acid, 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 50 mmol/l dithiothreitol, 5 mmol/l protease inhibitor cocktail and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF)]. Following homogenization for 5-6 repetitions, cell homogenates were centrifuged at 1,000 × g for 10 min at 4°C and the supernatant was collected. The supernatant was then centrifuged at 7,000 × g for 10 min at 4°C. The pellet was collected and the supernatant was centrifuged at 15,000 × g for 10 min at 4°C. The pellet was collected and exposed to density gradient centrifugation using Nycodenz® (Axis-Shield, Oslo, Norway). The isolated mitochondria were then stored in a buffer solution at -80°C until required.

Protein extraction and western blot analysis. Cells were lysed in pre-chilled lysis buffer (50 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 1 mM PMSF (Gibco-BRL), 10 mg/ml aprotinin (Santa Cruz Biotechnology, Inc.), 10 mg/ml leupeptin (Amresco, Solon, OH, USA) and 1% Nonidet P-40 (Sigma; pH 7.4). Lysates were centrifuged at 12,000 × g for 20 min, after which the supernatant was collected and mixed with loading buffer (65 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol and 6 M urea; Millipore, Billerica, MA, USA). Total protein concentrations were measured using a Pierce™ BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA). The electrophoresis buffer was supplemented with β-mercaptoethanol (Solarbio, Beijing, China) and bromophenol blue (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were separated using 12% SDS-PAGE, transferred onto a polyvinylidene fluoride membrane (Bio-Rad), and incubated with primary antibodies at 4°C overnight. Protein bands were detected using an enhanced chemiluminescence method and the intensity of the protein bands were analyzed using a gel image-analysis system (Molecular Imager Gel DocTM XR+ system; Bio-Rad).

Total RNA extraction and quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the cells in each group using an RNAiso™ Plus kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. Total RNA was subsequently quantified. The upstream primer for the δ-opioid receptor was 5′-ACCAAGATCTGGTGTTCCT-3′, and the downstream primer was 5′-CGATGCAGATGGATGGATG-3′. The upstream primer for the internal control, β-actin, was 5′-AAGGAAGGCTGGAAGAGTGC-3′, and the downstream primer was 5′-CTGGGACGACATGGAGAAAG-3′. qPCR was performed using a Takara RNA PCR kit (AMV) Version 3.0 (Takara Bio Inc.). The reaction conditions were as follows: Pre-denaturation at 94°C for 2 min followed by 31 cycles of denaturation at 94°C for 30 sec, annealing at 94°C for 30 sec and extension at 72°C for 30 sec, then a final extension at 72°C for 8 min. PCR amplicons were separated on 1.5% agarose and analyzed using a gel imaging analysis system (Molecular Imager Gel DocTM XR+ system; Bio-Rad).

Caspase detection. The activity of caspase-3 was determined using the Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China). To evaluate the activity of caspase-3, cell lysates were prepared following their respective treatment with various designated treatments. Assays were performed on 96-well microtitre plates by incubating 10 µl protein of cell lysate per sample in 80 µl reaction buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 10% glycerol] containing 10 µl caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37°C for 4 h.

Statistical analysis. All results are presented as the mean ± standard error of the mean. The effects of the chemicals at different concentrations were analyzed using the analysis of variance method. Differences between groups were analyzed using the unpaired Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

H₂O₂-induced apoptosis in human liver cancer cells and the protective role of δ-opioid receptor activation. In human liver cancer cells cultured in media containing H₂O₂ for 12 h, the number of adhesive cells was observed to decrease and the morphology of the cells was observed to become round- or oval-shaped. These features were enhanced in a H₂O₂ concentration-dependent manner. Flow cytometry revealed that upon H₂O₂ treatment, the number of apoptotic cells increased in a concentration-dependent manner (Fig. 1A). The absorbance (A)₅₇₀nm value of the liver cancer cells was also found to decrease in a concentration-dependent manner following H₂O₂ addition (Fig. 1B).

Caspase family members have key roles in apoptosis. In the present study, caspase-3 and -8 expression were observed to increase in a concentration-dependent manner with H₂O₂ treatment, which was consistent with the increase in apoptosis observed (Fig. 1C). In order to investigate whether the H₂O₂-induced apoptosis was associated with the mitochondrial pathway in human liver cancer cells, mitochondrial and cytoplasmic levels of cytochrome c, as well as changes in mitochondrial membrane potential, were analyzed. H₂O₂ was found to significantly decrease the mitochondrial membrane potential (Fig. 1D). Furthermore, levels of cytochrome c in the cytoplasm were observed to significantly increase.
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(Fig. 1E and G), while those in the mitochondria gradually decreased (Fig. 1F and G). These findings suggested that H₂O₂-induced human liver cancer cell apoptosis proceeded through the mitochondrial pathway.

Upon treatment with 200 mM H₂O₂, the addition of various concentrations of DADLE was found to increase the A₅₇₀nm value of liver cancer cells to various degrees. Increases in DADLE concentration from 0.01 to 1 µM were observed to increase the A₅₇₀nm value of liver cells in a dose-dependent manner. However, at concentrations >1 µM, no further increases were observed in the A₅₇₀nm value of liver cancer cells. These findings suggested that DADLE had a dose-dependent protective effect against H₂O₂-induced apoptosis in human liver cancer cells, with a maximum effect at 1 µM (Fig. 1H).

Effect of activated δ-opioid receptors on human liver cancer cell apoptosis. To study the effect of activated δ-opioid receptors on human liver cancer cell apoptosis, apoptosis was induced in liver cancer cells using H₂O₂. Cells were then treated with 1 µM DADLE, a specific δ-opioid receptor agonist. Annexin V-FITC/PI double-staining and flow cytometry revealed that the apoptosis rate of the cells treated with 200 mM H₂O₂ for 12 h was significantly increased compared with that in the control group. Furthermore, upon

Figure 1. Treatment of human liver cancer cells with various doses of H₂O₂ (0, 10, 50, 100, 200 and 400 mM) for 12 h. (A) Detection of apoptosis using Annexin V/propidium iodide double labeling. (B) MTT assay to assess cell viability. (C) Detection of caspase-3 and -8 activities. (D) Analysis of changes in mitochondrial membrane potentials using JC-1 staining and flow cytometry. (E-G) Analysis of cytoplasmic and mitochondrial cyt c expression using western blot analysis. (H) MTT assay to assess liver cancer cell survival following treatment with increasing concentrations of DADLE (0.01, 0.1, 1.0 and 10 µM) with H₂O₂ treatment. *P<0.05 vs. control group. Data are representative of three independent experiments. DADLE, [d-Ala²,d-Leu⁵] enkephalin; cyt c, cytochrome c; HSP, heat shock protein; OD, optical density.
DADLE-induced δ-opioid receptor activation, the apoptosis rate was found to significantly decrease compared with the cells treated solely with H₂O₂. Moreover, when δ-opioid receptor activation was inhibited using 10 µM naltrindole, a δ-opioid receptor antagonist, the DADLE-induced protective effect was reverted (Fig. 2A and B). Caspase-3 and -8 expression was observed to increase rapidly following H₂O₂ treatment. δ-opioid receptor activation was found to significantly downregulate cytoplasmic caspase-3 and -8 levels, and naltrindole was observed to inhibit this protective δ-opioid receptor-induced effect (Fig. 2C). These findings suggested that δ-receptor activation significantly inhibits H₂O₂-induced apoptosis in human liver cancer cells.

Activated δ-opioid receptors inhibit human liver cancer cell apoptosis through the mitochondrial pathway. To investigate whether the molecular mechanisms underlying the inhibition of liver cancer cell apoptosis by activated δ-opioid receptors are associated with the mitochondrial pathway, changes in mitochondrial membrane potential were analyzed. H₂O₂ treatment was found to gradually decrease the mitochondrial membrane potential in liver cancer cells. Concurrent δ-opioid receptor activation and H₂O₂ treatment had no significant effect on the mitochondrial membrane potential (Fig. 3A). Cytochrome c is released from mitochondria into the cytoplasm during apoptosis; therefore, western blot analysis was performed to investigate the cytoplasmic and mitochondrial cytochrome c levels. Compared with the cells treated with H₂O₂ alone, upon activation of the δ-opioid receptors with H₂O₂ treatment, cytoplasmic cytochrome c levels were observed to decrease and mitochondrial cytochrome c levels were found to increase (Fig. 3B and C). Furthermore, δ-opioid receptor activation was observed to increase cytoplasmic Bax and decrease Bcl-2 expression (Fig. 3D and E). These findings suggested that DADLE may activate δ-opioid receptors at the surface of the plasma membrane in liver cancer cells in order to stabilize mitochondrial membrane potentials and inhibit H₂O₂-induced apoptosis in human liver cancer cells.

δ-opioid receptors affect human liver cancer cell apoptosis through the PKC/ERK pathway. In order to investigate whether δ-opioid receptors activate the PKC/ERK signaling pathway and whether inhibiting this signaling pathway alters the effect of δ-opioid receptors on human liver cancer cell apoptosis, the phosphorylation levels of PKC and ERK were investigated. Following δ-opioid receptor activation, phosphorylated PKC and ERK were observed to be significantly increased in the cytoplasm of human liver cancer cells (Fig. 4A and B), suggesting that δ-opioid receptor activation may lead to phosphorylation of PKC and ERK. It has previously been reported that δ-opioid receptor activation inhibits apoptosis in human liver cancer cells (23). However, in the present study, inhibiting treatment with 10 µM naltrindole abolished the protective effect of DADLE on liver cancer cell apoptosis (Fig. 2A and B). Furthermore, the phosphorylation levels of PKC and ERK were significantly decreased when δ-opioid receptor activation was inhibited using 10 µM naltrindole (Fig. 4C), suggesting that δ-opioid receptor activation is required for the phosphorylation of PKC and ERK. These findings suggest that δ-opioid receptor activation inhibits apoptosis in human liver cancer cells through the PKC/ERK signaling pathway.
the PKC pathway was found to increase apoptosis and inhibit cell proliferation, regardless of the δ-opioid receptor activation status. Inhibition of the ERK pathway was observed to have the same effect (Fig. 4C and D). These findings suggested that the PKC and ERK pathways are involved in mediating the protective effect of δ-opioid receptors on H₂O₂-induced human liver cancer cell apoptosis.

**δ-opioid receptors are highly expressed in human liver cancer cells.** To assess whether δ-opioid receptors are expressed in human liver cancer tissues and cells, and whether they have a role in carcinogenesis, qPCR analysis was used to assess δ-opioid receptor mRNA expression in 50 liver cancer samples. δ-opioid receptors were found to be expressed in the 50 liver cancer samples, with the expression levels observed to be higher than those in the adjacent normal liver tissue. δ-opioid receptor mRNA expression was also analyzed in several liver cancer cell lines (Fig. 5A). Western blot analysis further revealed that the protein expression of the δ-opioid receptor was higher in liver cancer tissue than in the adjacent normal tissue. In addition, δ-opioid receptor protein expression was found to be higher in the liver cancer cell lines than in the normal liver cell lines (Fig. 5B). These findings indicate that δ-opioid receptors are highly expressed in human liver cancer tissues and cells and have an important role in liver cancer cell proliferation.

**Discussion**

The present study investigated the effect of activated δ-opioid receptors on liver cancer cell apoptosis. Liver cancer cell apoptosis has an important role in liver tumorigenesis and liver...
cancer progression. Thus, identifying the mechanisms underlying liver cancer cell apoptosis, as well as promoting liver cancer cell apoptosis, may have important consequences with regard to treating liver cancer and protecting liver function. It has been shown that H$_2$O$_2$ is capable of inducing histological changes, including changes in cell morphology, cytoskeletal rearrangement, intracellular accumulation of reactive oxygen species and changes in mitochondrial function; all of which promote apoptosis (24). H$_2$O$_2$ is commonly used to induce apoptosis (25,26) and in the present study, H$_2$O$_2$ was found to significantly induce apoptosis in human liver cancer cells in a time-dependent manner. H$_2$O$_2$ treatment also decreased the mitochondrial membrane potential, which was followed by the activation and release of cytochrome c into the cytoplasm and an increase in caspase-3 expression. These findings indicated that H$_2$O$_2$-induced apoptosis is achieved through the mitochondrial pathway.

Opioid receptors are widely expressed across human tissues. A previous study by our group reported that δ-opioid receptors are expressed in normal liver tissues, particularly in liver cancer tissues. δ-opioid receptors predominantly participate in cell survival and proliferation. Su (27) showed that δ-opioid receptors have a protective role in the liver, with δ-opioid receptors reported to antagonize cholestasis in animal models (28). The previous study by our group showed that δ-opioid receptors decreased normal liver cell
apoptosis (5). In addition, endogenous opioid peptides have been found to promote liver cancer cell proliferation (8). This protective effect occurs through the activation of δ-opioid receptors at the plasma membrane. The present study observed that δ-receptor activation inhibited liver cancer cell apoptosis and downregulated caspase-3 expression. This may be a protective mechanism for maintaining liver cancer cell self-repair functions.

Numerous studies have demonstrated that opioid receptors activate pertussis toxin-sensitive G-protein (29) and ATP-sensitive potassium channel (30) signaling pathways in order to exert their cellular functions. Furthermore, Zhao et al (31) showed that opioid receptors activate ERK in order to promote cell survival and proliferation. The present study indicated that δ-opioid receptor activation increased PKC and ERK expression. Activated PKC has been reported to inhibit various types of apoptosis (32-34). The previous study by our group reported that δ-opioid receptors were involved in cell proliferation and apoptosis in hepatic ischemia reperfusion injuries through activating PKC (5). Thus, PKC also participates in liver cancer cell proliferation and apoptosis. In the present study, δ receptor activation was found to significantly increase PKC expression. Furthermore, inhibition of PKC was observed to increase liver cancer cell apoptosis, independent of δ-opioid receptor activation status. Previous studies have shown that activated δ-opioid receptors activate the ERK signaling pathway (35,36). ERK is a MAPK which is involved in cell proliferation, transformation and differentiation. Activated ERK activates transcription through the phosphorylation of p90 ribosomal S6 kinase and mitogen and stress activated protein kinase, as well as the transcription factors ELK-1 and signal transducer and activator of transcription 3, thereby inducing cell growth, proliferation and differentiation. The present study found that following δ-opioid receptor activation, ERK phosphorylation levels significantly increased and liver cancer cell apoptosis decreased. However, inhibition of the ERK pathway significantly increased apoptosis in the liver cancer cells, regardless of δ-opioid receptor activation. These findings suggested that PKC and ERK participated in the regulation of human liver cancer cell apoptosis through the δ-opioid receptor.

A previous study by our group identified that H$_2$O$_2$ induced apoptosis through the mitochondrial pathway, which was similar to the findings of Li et al (37). In the present study, H$_2$O$_2$ stimulation was found decrease mitochondrial membrane potentials, increase cytochrome c levels, increase the translocation of Bax from the cytoplasm to mitochondria and induce apoptosis. However, upon activation of the δ-opioid receptors, H$_2$O$_2$-induced apoptosis was inhibited. These findings suggested that the protective role of δ-opioid receptors in liver cancer cells was achieved through the mitochondrial pathway.

In conclusion, the present study demonstrated that H$_2$O$_2$-induced human liver cancer apoptosis occurred through the mitochondrial pathway. Furthermore, the activation of δ-opioid receptors was found to protect cells from undergoing apoptosis through the mitochondrial pathway. In addition, the protective effect of δ-opioid receptors on H$_2$O$_2$-induced apoptosis was found to be mediated through the PKC, ERK and mitochondrial pathways. Further elucidation of this apoptotic mechanism is important for understanding the role of δ-opioid receptors in human liver cancer cell apoptosis and may have important implications for liver cancer treatment.

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**References**