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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. H_2O_2 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 H_2O_2-induced apoptosis was mediated through the mitochondrial pathway. Of note, activated δ-opioid receptors were observed to inhibit H_2O_2-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.

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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).

H_2O_2 is commonly used to induce apoptosis (10). In the present study, different concentrations of H_2O_2 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 H_2O_2 model of apoptosis mimics the physiological conditions of hepatic ischemia and hypoxia. Apoptosis proceeds via two major pathways: The death receptor pathway and the mitochondrial 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, H_2O_2 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 cytoplasm. Following external stimulation, PKC is translocated from the cytoplasm to the plasma membrane and is activated. The PKC signaling pathway is involved in various biological activities, in which it mediates proliferation and differentiation 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 identified 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 demonstrated 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, H_2O_2 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 interrelation 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-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine 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 phosphorylated 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 $1x10^6$ cells/ml in T25 cell-culture flasks and cultured in Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% FBS, penicillin and streptomycin in an incubator with 95% O_2 and 5% CO_2.

Experimental treatment. Liver cancer cells were cultured for 12 h. Except for those in the control group, cells were treated with various concentrations of H_2O_2 (10, 50, 100, 200 and 400 mM) for 12 h. While under H_2O_2 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 H_2O_2 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 culture 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 wavelength 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 x g for 10 min at 4˚C and the supernatant was collected. The supernatant was then centrifuged at 7,000 x g for 10 min at 4˚C. The pellet was collected and the supernatant was centrifuged at 15,000 x 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 x 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'-ACCAAGATCTGCGTGTTCCT-3', and the downstream primer was 5'-CGATGACGAAGATGTGGATG-3'. The upstream primer for the internal control, β-actin, was 5'-AAGGAAGGCTGGAAGAGTGC-3', and the downstream primer was 5'-CTGGGACGACATGGAGAAAA-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 Nad 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

H_2O_2-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 H_2O_2 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 H_2O_2 concentration-dependent manner. Flow cytometry revealed that upon H_2O_2 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 H_2O_2 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 H_2O_2 treatment, which was consistent with the increase in apoptosis observed (Fig. 1C). In order to investigate whether the H_2O_2-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. H_2O_2 was found to significantly decrease the mitochondrial membrane potential (Fig. 1D). Furthermore, levels of cytochrome *c* in the cytoplasm were observed to significantly increase

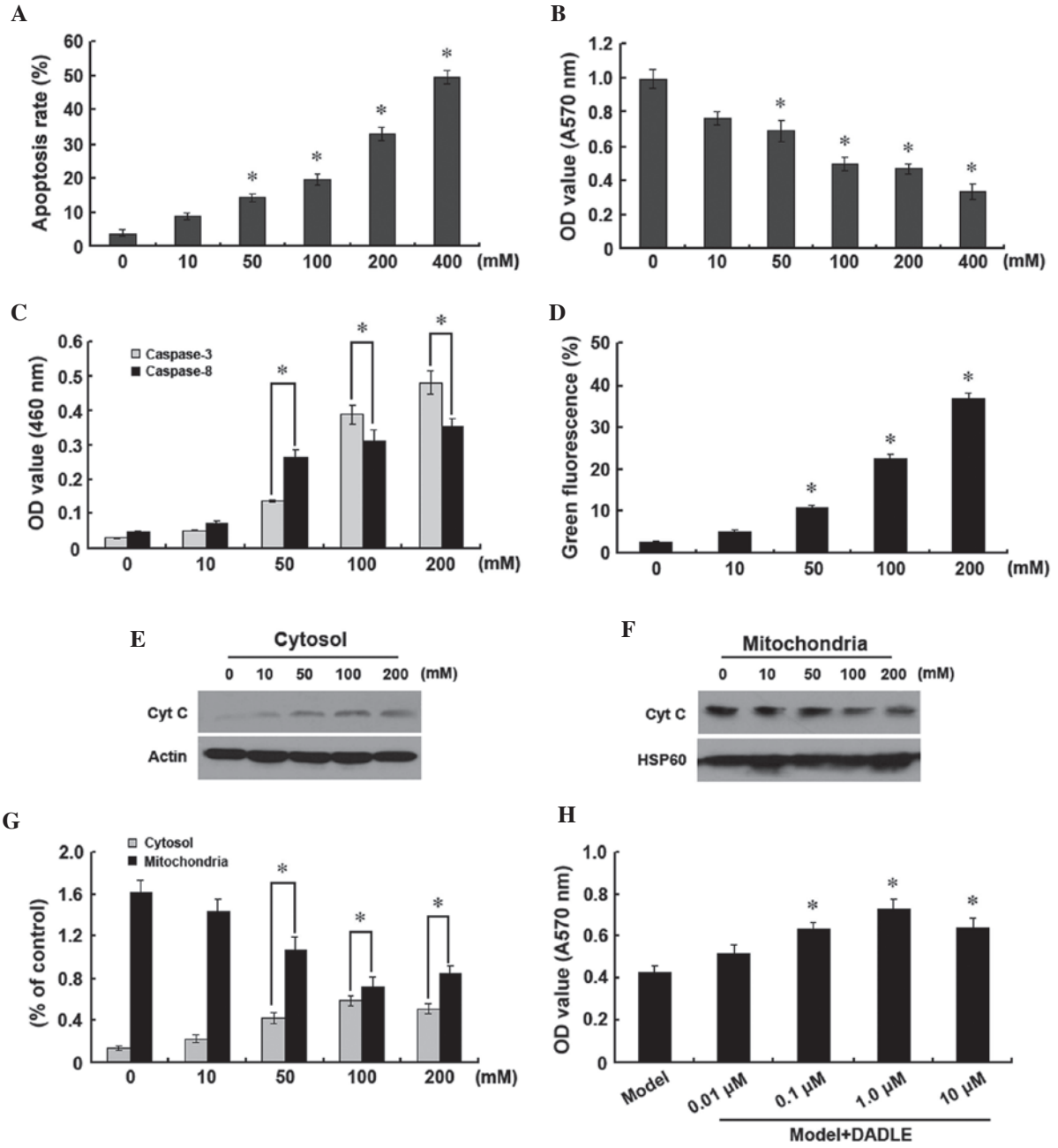

Figure 1. Treatment of human liver cancer cells with various doses of H_2O_2 (0, 10, 50, 100, 200 and 400 mM) for 12 h. (A) Detection of apoptosis using Annexin V/propidium iodide double labeling. (B) MTT assay to assess cell viability. (C) Detection of caspase-3 and -8 activities. (D) Analysis of changes 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 H_2O_2 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.

(Fig. 1E and G), while those in the mitochondria gradually decreased (Fig. 1F and G). These findings suggested that H_2O_2-induced human liver cancer cell apoptosis proceeded through the mitochondrial pathway.

Upon treatment with 200 mM H_2O_2, the addition of various concentrations of DADLE was found to increase the A_{570nm} value of liver cancer cells to various degrees. Increases in DADLE concentration from 0.01 to 1 μM were observed to increase the A_{570nm} value of liver cells in a dose-dependent manner. However, at concentrations >1 μM, no further increases were observed in the A_{570nm} value of liver cancer cells. These findings suggested that DADLE had a dose-dependent protective effect against H_2O_2-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 H_2O_2. 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 H_2O_2 for 12 h was significantly increased compared with that in the control group. Furthermore, upon

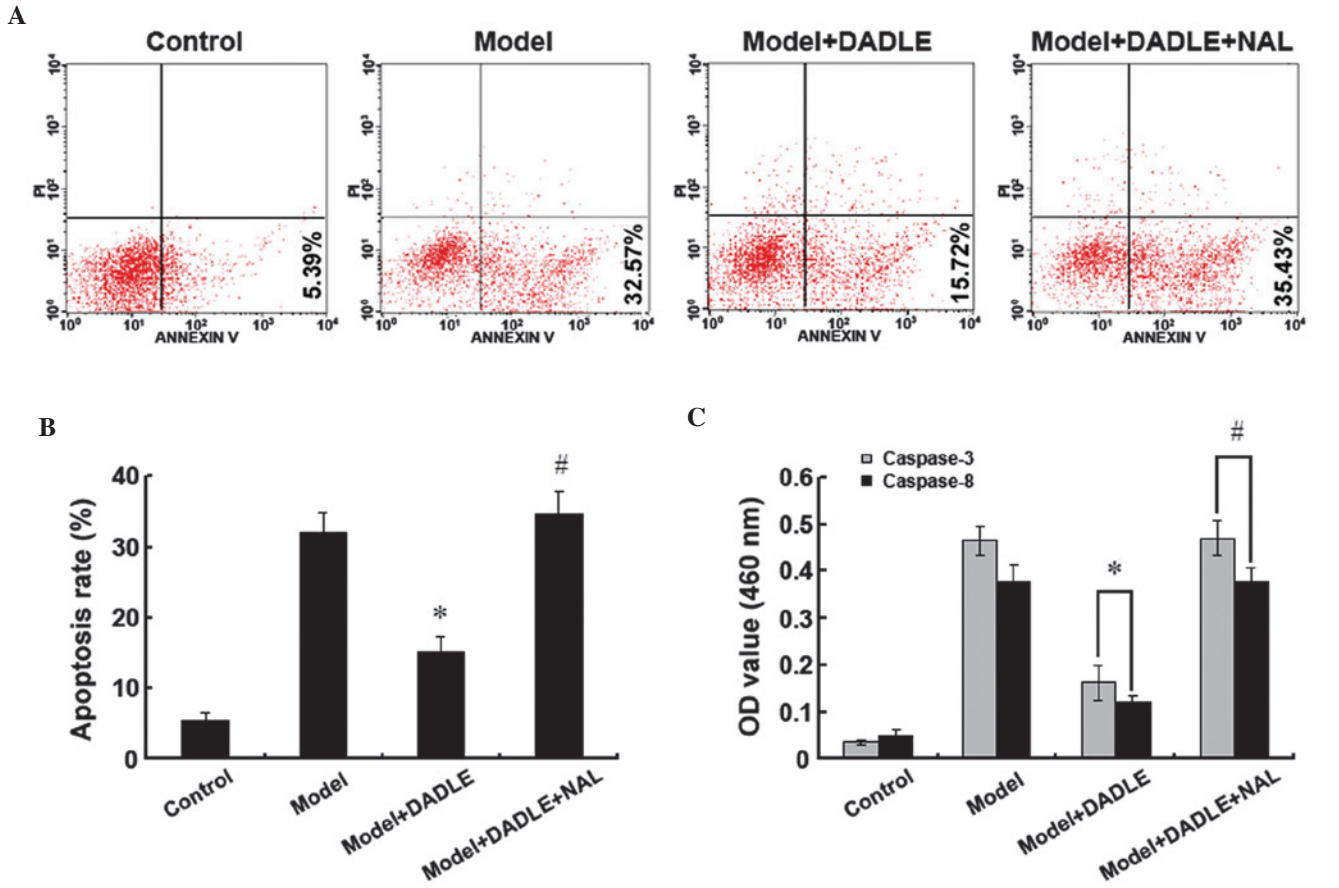

Figure 2. Treatment of cells with 200 mM H_2O_2 combined with 1 μM DADLE or 10 μM NAL. (A and B) Flow cytometric analysis of liver cancer cell apoptosis. (C) Detection of changes in caspase-3 and -8 activities. *P<0.05 vs. the model group; #P<0.05 vs. the model + DADLE group. Data are representative of three independent experiments. NAL, naltrindole; DADLE, [D-Ala2, D-Leu5] enkephalin; OD, optical density.

DADLE-induced δ-opioid receptor activation, the apoptosis rate was found to significantly decrease compared with the cells treated solely with H_2O_2. 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 H_2O_2 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 H_2O_2-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. H_2O_2 treatment was found to gradually decrease the mitochondrial membrane potential in liver cancer cells. Concurrent δ-opioid receptor activation and H_2O_2 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 H_2O_2 alone, upon activation of the δ-opioid receptors with H_2O_2 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 H_2O_2-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

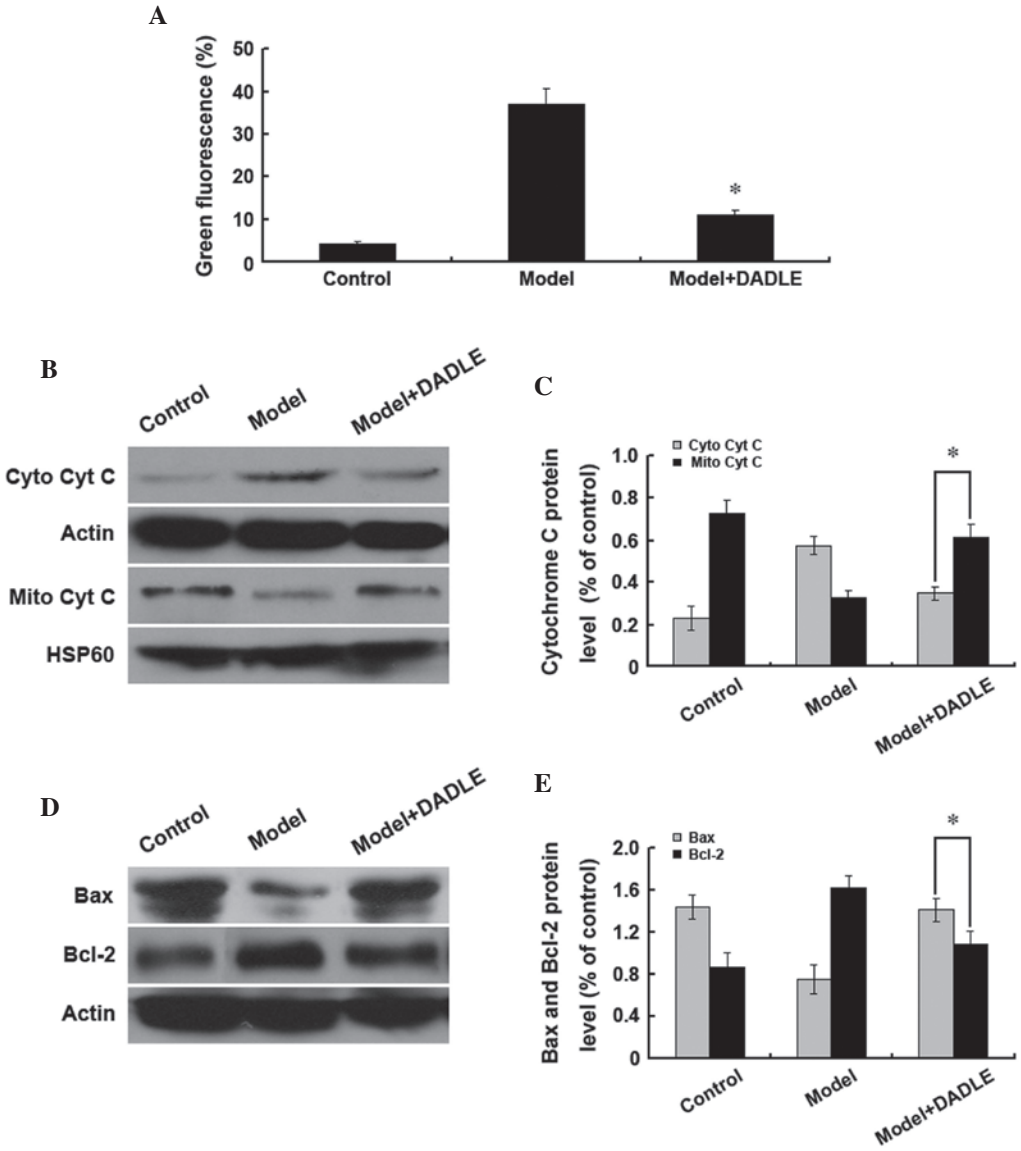

Figure 3. δ-opioid receptor activation inhibits human liver cancer cell apoptosis through the mitochondrial pathway. Cells were treated with 200 mM H_2O_2 and the δ-opioid receptor agonist DADLE (1 μM). (A) JC-1 staining and flow cytometric analysis of changes in mitochondrial membrane potential. *P<0.05 vs. the model group. (B-E) Western blot analysis of changes in cytoplasmic and mitochondrial (B and C) cyt *c* protein expression and (D and E) Bax and Bcl-2 protein expression. Data are representative of three idependent experiments. DADLE, [D-Ala2, D-Leu5] enkephalin; cyt *c*, cytochrome *c*; Mito, mitochondrial; cyto, cytoplasmic; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; HSP, heat shock protein; JC-1, 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide.

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 H_2O_2-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

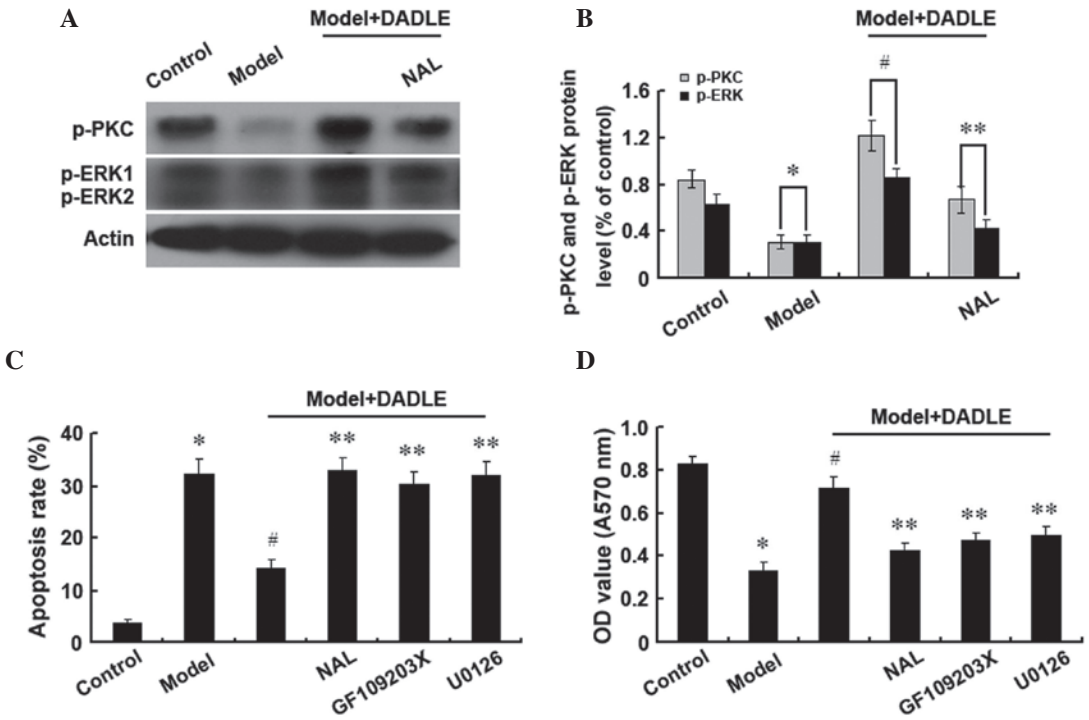

Figure 4. Effect of δ-opioid receptor activation on human liver cancer cell apoptosis following inhibition of the PKC and ERK signaling pathways. Cells were treated with the δ-receptor agonist DADLE (1 μM) and either the PKC inhibitor GF109203X (1 μM) or the ERK inhibitor U1026 (10 μM). (A and B) Levels of phosphorylated PKC and ERK were detected using western blot analysis. (C) Flow cytometric analysis of liver cancer cell apoptosis. (D) MTT assay to assess liver cancer cell viability. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the model group; **P<0.05 vs. the model + DADLE group. Data are representative of three independent experiments. DADLE, [D-Ala2, D-Leu5] enkephalin; NAL, naltrindole; p-, phosphorylated; PKC, protein kinase C; ERK, extracellular-signal-regulated kinase; OD, optical density.

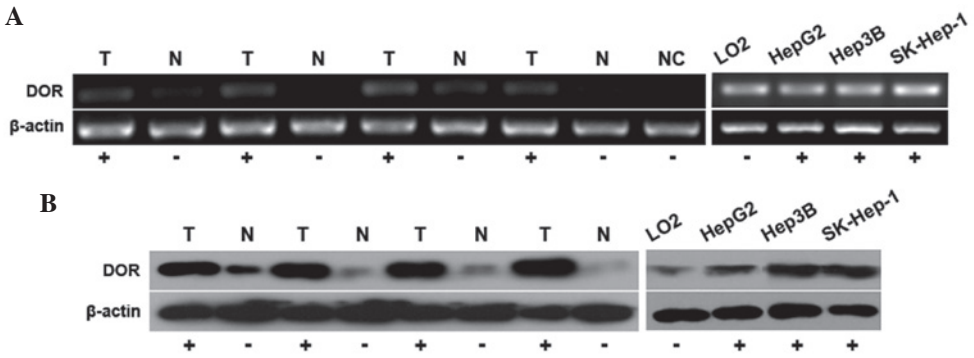

Figure 5. δ-opioid receptor expression in human liver cancer tissues and liver cancer cell lines. (A) Quantitative polymerase chain reaction and (B) western blot analyses of δ-opioid receptor expression in human liver cancer tissues, adjacent tissues, liver cancer cell lines and normal liver cells. Data are representative of three independent experiments. DOR, δ-opioid receptor; T, tumor; N, normal; NC, negative control.

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_2O_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_2O_2 is commonly used to induce apoptosis (25,26) and in the present study, H_2O_2 was found to significantly induce apoptosis in human liver cancer cells in a time-dependent manner. H_2O_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_2O_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_2O_2 induced apoptosis through the mitochondrial pathway, which was similar to the findings of Li *et al* (37). In the present study, H_2O_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_2O_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_2O_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_2O_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.

Acknowledgements

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