Activation of the δ-opioid receptor inhibits serum deprivation-induced apoptosis of human liver cells via the activation of PKC and the mitochondrial pathway

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Received June 27, 2011; Accepted August 8, 2011

DOI: 10.3892/ijmm.2011.784

Abstract. Apoptosis of human liver cells is commonly found in liver diseases and liver surgery and directly affects their prognosis. Recent studies have found that δ-opioid receptors, abundant in the membranes of hepatic cells, participate in the oncogenesis and progression of liver tumors, viral hepatitis, liver cirrhosis and other diseases. The purpose of this study was to analyze the effect of the activation of the δ-opioid receptor on liver cell apoptosis and explore its relationship with PKC and the mitochondrial pathway. Hepatic cells were serum-deprived to induce apoptosis in vitro. During the period of apoptosis, mitochondrial membrane potential decreased, protein levels of cytosolic cytochrome c increased and the expression of Bcl-2 decreased, indicating that apoptosis was specifically induced by the mitochondrial pathway. Importantly, activation of δ-opioid receptors reversed the apoptotic state of hepatic cells. Following δ-opioid receptor activation, the mitochondrial membrane potential remained stable, and the expression of cytosolic cytochrome c and Bax decreased. These data suggest that δ-opioid receptor activation specifically inhibits the mitochondrial apoptotic pathway. In addition, activation of the δ-opioid receptor apparently increased the levels of PKC; blocking the PKC pathway led to increased apoptosis of liver cells, which was not affected by the activation of δ-opioid receptor. Blocking the PKC pathway led to increased apoptosis of liver cells, which was associated with δ-opioid receptor activation. Therefore, the PKC pathway is involved in the anti-apoptotic effects of the δ-opioid receptor on liver cells.

Introduction

Liver cell apoptosis plays an important part in the regulation of normal liver function, which is mediated by a number of signal transduction pathways (1). Yet, apoptosis serves as the cytological basis for the generation of many liver diseases (2-4). Therefore, reducing non-physiological apoptosis in hepatocytes has important clinical significance for protecting hepatic structure and function.

δ-opioid receptor belongs to the superfamily of opioid receptors, which are widely expressed throughout the human body. Studies have found that the agonist of the δ-opioid receptor stimulates the proliferation of neonatal rat cardiac myocytes (5) and mimics the protective effects of ischemic preconditioning on heart and brain tissue (6,7). These findings have demonstrated that the δ-opioid receptor plays a significant role in the growth and maintenance of cells. In addition to the central nervous system and heart, δ-opioid receptors are abundantly expressed in the liver (8,9). It has been recently discovered that the δ-opioid receptor affects the generation and progression of hepatic tumors, viral hepatitis, liver cirrhosis and other diseases (10,11).

Ischemia and hypoxia are the most commonly used methods to induce apoptosis of in vitro cultured cells (12). In this study, serum was removed from cell culture medium, and cells were grown in serum-free conditions for specified periods of time. The lack of growth factors in medium causes cells to undergo apoptosis (13,14). This cellular apoptosis model closely mimics the physiologic state of ischemia and hypoxia in the liver. Cellular apoptosis occurs mainly through two pathways: the death receptor pathway and the mitochondrial pathway (15). The mitochondrial apoptotic pathway is initiated by altered mitochondrial structure and function, particularly the decline of the mitochondrial transmembrane potential. Cytochrome c
is then abundantly released from mitochondria to trigger a large apoptotic protease cascade, which ultimately activates caspase-3 and leads to the apoptosis of cells. Studies have shown that the δ-opioid receptor mediates protective effects on myocardial and nerve cells through inhibition of the mitochondrial apoptotic pathway (16,17). In this study, we found that serum deprivation induced apoptosis of human liver cells through the mitochondrial pathway. Therefore, it is questionable whether the anti-apoptotic effect of the δ-opioid receptor on liver cells is associated with the mitochondrial pathway.

Protein kinase C (PKC) is a large class of threonine kinases widely expressed in human cells. In the resting state, intracellular PKC is localized in the cytoplasm in the inactive form. Following stimulation by external factors, PKC translocates from the cytoplasm to the plasma membrane to become activated. The PKC signaling pathway is implicated in a wide range of biological activities and specifically mediates the proliferation and differentiation of a variety of cells. Studies have found that PKC is involved in the preconditioning protection of hepatic ischemia (18). The proliferation and apoptosis of normal cells and hepatic tumor cells closely correlate with PKC (19-22). Recent findings have shown that opioid receptors share a common pathway with the phosphorylation of PKC. In an isolated heart model, the cardioprotective effects of morphine were blocked by the PKC inhibitor, chelerythrine, (23), indicating that PKC mediates the pretreatment protective effect of morphine. Therefore, the intracellular signal transduction pathways activated by the stimulation of opioid receptors include the PLC-PKC pathway, the PTX-sensitive G protein-PLC-IP3-Ca2+ pathway and the AC-cAMP pathway.

In the present research, using in vitro cultured human liver cells, serum deprivation as a condition was adopted to induce the apoptosis of liver cells in order to investigate the effects of the δ-opioid receptor on the apoptosis of human liver cells and its relationship with PKC and the mitochondrial pathway.

Materials and methods

Materials. Surgically resected human liver tissues were provided by the Third Department of General Surgery, The Second Affiliated Hospital of Dalian Medical University, and approval was obtained from the ethics committee. Materials used included the following: DADLE, Naltrindole, GF109203X and I, IV collagenase and Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA); fetal bovine serum (Gibco, USA); MTT (Sigma); the Annexin V-FITC kit (Bio-Rad, Hercules, CA, USA); PKC, Bcl-2 and Bax antibodies (Santa Cruz Biotechnology); and the cytochrome c and caspase-3 antibodies (Cell Signaling).

Isolation and culture of human liver cells. Human liver tissues were obtained from 14 male patients with partial hepatectomy, as approved by the ethics committee. Human hepatocytes were obtained from 14 male patients with partial hepatectomy, and approval was obtained from the ethics committee. Human hepatocytes were isolated from 15% heat-inactivated fetal bovine serum, 84% DMEM and 1% penicillin-streptomycin solution.

Experimental treatments. Human liver cells were cultured for 48 h, and the medium of each group apart from the control group was replaced with serum-free DMEM and cultured further. The cells were collected at different time points (0, 24, 48 h) for analysis. During serum deprivation, the intervention groups were treated with different concentrations of DADLE (0.01, 0.1, 1.0 and 10 µM) and the PKC antagonist GF109203X (10 µM) for 48 h.

Cell viability. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human liver cells were serum-deprived for 48 h. Thereafter, 20 µl of sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide stock solution (5 mg/ml) in PBS (pH 7.4) was added to each well, and the cells were incubated for 4 h at 37°C to allow the yellow dye to be transformed into blue crystals. The unreacted dye was removed by aspiration, and 200 µl of DMSO was added to each well to dissolve the dark blue crystals. Finally, the optical density was measured with a microtiter plate reader at 570 nm. The spectrophotometer was normalized using culture medium without cells. Cell viability (%) relative to control wells containing culture medium was calculated by [(A)test/(A)control]. Eight replicates were prepared for each condition.

Annexin V-FITC/PI double-labeled detection of apoptosis. The protocol was based on the use of fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) staining, according to the manufacturer's instructions (Annexin V-FITC kit; Bio-Rad). Analysis by flow cytometry was carried out to differentiate the types of cell death. Cells that were Annexin V-positive and PI-negative were classified as apoptotic or early-stage apoptotic cells. Briefly, cells were digested with 0.25% trypsin, washed three or four times and then stained for 15-20 min until the cells were completely digested. After cells were digested, they were washed with PBS. Unfixed cells were stained by adding the Annexin V-FITC reaction mixture (10 µl Annexin V-FITC, 5 µl propidium iodide) and incubated at room temperature for 15 min in the dark. The stained cells were subjected to flow cytometric analysis with a FACSCalibur (Becton-Dickinson, USA).

The isolation and purification of mitochondria. Liver cells from the different groups were collected and pre-cooled in extraction buffer (0.2 M mannitol, 50 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, pH 7.4, 50 mM DTT, 5 mM cocktail, 1 mM PMSF) was added. Then, the cells were homogenized for 5-6 strokes and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was collected and centrifuged at 15,000 x g for 10 min, and the precipitate was reserved. The Nycodenz density gradient centrifugation was performed according to Sun et al (26). Finally, the mitochondria were placed in 1-2 ml of buffer and preserved at -80°C.

JC-1 staining and flow cytometric analysis of mitochondrial membrane potential. After the density of the mitochondria
isolated from the previous method was adjusted, 10 g/ml JC-1 solution (dissolved with DMSO) was added, and the solution was fully mixed and incubated in a 5% CO₂ incubator in the dark for 30 min at 37°C. The excess unbound dye was washed off twice with 1X assay buffer, and the precipitate was resuspended with PBS. The cells were analyzed by flow cytometry (BD Company, US) at an emission wavelength of 488 nm; each sample acquired 1×10⁶ cells. JC-1 monomers and aggregates were visualized on FL1 and FL2 detectors, respectively. As the emission of JC-1 had a dual wavelength, electronic compensation was applied to modify the overlap of the green fluorescence (monomer) and red fluorescence (polymer). FL1-H and FL2-H represented the fluorescence intensity of red and green, respectively. Quantitative analysis was performed by CellQuest Analysis software.

**Protein extraction and Western blot analysis.** Cells were washed once with ice-cold PBS containing 100 mM sodium orthovanadate and solubilized in lysis buffer (50 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% Nonidet P-40; pH 7.4). After centrifugation at 12,000 x g for 20 min, the supernatant was collected. Cells were dissolved in sample buffer containing 65 mM Tris-HCl (pH 6.8), 3% SDS, 10% glycerol and 6 M urea. After determination of the protein concentration (BCA kit; Pierce, Rockford, IL, USA), β-mercaptoethanol and bromophenol blue were added to the sample buffer for electrophoresis. The protein was separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transfiblated to polyvinylidene difluoride membranes (Bio-Rad). The blots were incubated at 4°C overnight with antibodies, and the resulting bands were detected using enhanced chemiluminescence. Intensities of the bands were quantified using an image-analysis system.

**RT-PCR.** Total RNA from the cells of the different groups was prepared by using RNAisoTM Plus (Takara), and the concentration of RNA was evaluated using a spectrophotometer. The reverse transcription and PCR were performed using Takara RNA PCR kit (AMV) ver. 3.0 according to the protocols of the manufacturer. The primers for amplification were as follows: PKC, forward primer, 5’-TGAATCCTCAGTGGAATGAT-3’ and reverse primer, 5’-GGTGGCTTCTCTGCTTGAA-3’; β-actin, forward primer, 5’-AAGGAAGGCTGGAGAAGTG-3’ and reverse primer, 5’-CCTGGGACGCATGGGAGAAA-3’. The PCR reactions were subjected to the following cycle: denaturation (94°C, 30 sec), annealing (55°C for PKC, 61°C for β-actin, 30 sec) and extension (72°C, 30 sec); PKC and β-actin: 29 cycles.

**Statistical analysis.** All data are expressed as the mean ± SEM. To analyze the effects of drugs at different concentrations, analysis of variance (one-way ANOVA) was used. The unpaired Student’s t-test was used to test for differences between two groups. A P-value <0.05 was considered statistically significant.

**Results**

The effect of serum deprivation on the apoptosis of human liver cells. Human liver cells were serum-deprived to induce apoptosis. After 24 h of serum deprivation, the number of adherent liver cells decreased, and the cell morphology was round and oval, which became more evident over time. Flow cytometric analysis indicated the increased apoptosis of cells overtime (Fig. 1A and B). We observed an initial decline in mitochondrial potential at early time points of serum deprivation. With the prolongation of time, the mitochondrial potential decreased markedly (Fig. 1C and D). Caspase-3 is a crucial mediator of cellular apoptosis. The levels of caspase-3 increased with serum deprivation over time (Fig. 1E and F). This result indicates that serum deprivation leads to apoptosis of liver cells and that the strongest apoptotic response occurs at 48 h. The mitochondrial pathway is the classic pathway that mediates cell apoptosis. We examined the expression of cytochrome c and Bel-2 and the changes in mitochondrial potential to determine whether the liver cell apoptosis induced by serum deprivation is mediated by the mitochondrial pathway. As the expression of cytochrome c in the cytoplasm apparently increased (Fig. 1G and H), the expression of Bel-2 (Fig. 1K and L) decreased gradually. In contrast, cytochrome c inside the mitochondria decreased gradually (Fig. 1I and J). These results suggest that the apoptosis of human liver cells by serum deprivation occurs through the mitochondrial pathway.

The effect of δ-opioid receptor activation on the apoptosis of human liver cells. Increasing concentrations of DADLE were added to the culture medium at the time when the serum was deprived. The value of A570 nm in human liver cells increased to varying degrees. At concentrations of DADLE over 1.0 µM, the value of A570 nm no longer increased. Therefore, DADLE inhibits liver cell apoptosis in a concentration-dependent manner; the most prominent effect was observed at 1.0 µM (Fig. 2A). To determine the effects of δ-opioid receptor activation on the apoptosis of human liver cells, 1 µM of the δ-opioid receptor agonist DADLE and 1 µM of the δ-opioid receptor antagonist Naltrindole were added to the cell culture medium when apoptosis was induced. The cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. After 48 h of serum deprivation, the apoptotic rate of the liver cells was 23.72%, which was increased by 18.71% compared with the control group. After the δ-opioid receptor was activated by DADLE, the apoptosis rate was 16.62%, which was reduced by 7.1% compared with serum deprivation. When the δ-opioid receptor was blocked by Naltrindole, the apoptosis rate was increased by 27.19% (Fig. 2B and C). After serum deprivation, the level of caspase-3 rose rapidly, while after activation of the δ-opioid receptor, the level of caspase-3 tended to decline, which were blocked by Naltrindole (Fig. 2D and E). In conclusion, activation of the δ-opioid receptor suppresses liver cell apoptosis induced by serum deprivation.

The activation of the δ-opioid receptor inhibits apoptosis of human liver cells through the mitochondrial pathway. To verify the relationship between the protective function of the δ-opioid receptor on human liver cells and the mitochondrial pathway, alterations in the mitochondrial membrane potential were detected using JC-1 dye. JC-1 dye is a highly sensitive dye which detects changes in the mitochondrial membrane potential.
When mitochondrial membrane potential is high, JC-1 accumulates in the matrix of mitochondria by forming J-aggregates with red fluorescence. However, when mitochondrial membrane potential is low, JC-1 becomes a monomer with green fluorescence. In flow cytometric analysis, the lateral and longitudinal axes of coordinates represent the distribution of green and red fluorescence, respectively. The ratio of green and red fluorescence represents the depolarization.
tion percentage of mitochondria. The data reveal that serum deprivation induces the apparent decline of mitochondrial membrane potential, and the alteration of mitochondrial membrane potential occurs earlier than cell apoptosis. When the δ-opioid receptor was activated, there was no significant change in mitochondria membrane potential (Fig. 3A and B).

Cytochrome c release into the cytoplasm is another marker of apoptosis. To investigate the function of δ-opioid receptor activation on cytochrome c release, we analyzed the levels of cytochrome c in the mitochondria and cytoplasm by Western blot analysis. Compared with the levels induced by serum deprivation, the levels of cytosolic cytochrome c decreased with the activation of the δ-opioid receptor, while the levels of mitochondrial cytochrome c increased (Fig. 3C-F).

Upon induction of the apoptotic signal, Bax protein translocates from the cytoplasm to insert into the outer membrane of mitochondria, which alters the permeability of the mitochondrial outer membrane to promote the release of cytochrome c (27). δ-opioid receptor activation increased the levels of Bax protein in the cytoplasm and reduced its expression in the mito-
Our data revealed that DADLE activated the δ-opioid receptor on the membrane of liver cells, stabilized the mitochondrial membrane potential and prevented the release of cytochrome c from mitochondria and the translocation of cytosolic Bax. In this manner, serum deprivation-induced apoptosis of human liver cells was suppressed.
The function of δ-opioid receptor activation in the apoptosis of human liver cells under the blockade of the PKC pathway.

To analyze whether the blockade of the PKC pathway affects the protective function of the δ-opioid receptor on human liver cell apoptosis and whether the stimulation of δ-opioid receptor activates PKC, Western blot analysis and PR-PCR were used to detect the mRNA and protein expression of PKC. It was found that the δ-opioid receptor agonist DADLE increased both mRNA and protein expression of PKC markedly (Fig. 4A-D), indicating that stimulation of the δ-opioid receptor activates PKC. Moreover, these experiments demonstrated that the activation of the δ-opioid receptor inhibits the apoptosis of liver cells and down-regulates the expression of caspase-3. However, when the PKC pathway was blocked, caspase-3 expression levels increased in the presence of the activated δ-opioid receptor (Fig. 4E and F), indicating that the PKC pathway mediates the anti-apoptotic function of δ-opioid receptor activation. In addition, when the PKC pathway was blocked, the expression of cytochrome c was significantly increased. This indicated that there was a link between PKC and the mitochondrial pathway (Fig. 4G and H).

Discussion

In this study, we examined the effects of δ-opioid receptor activation on the apoptosis of human liver cells and explored the underlying mechanisms of protection. Apoptosis of human liver cells is an important feature of a variety of liver diseases, and it is often the cytological basis of disease occurrence. Therefore, clarifying the underlying mechanisms of apoptosis...
and reducing non-physiological levels of apoptosis in hepatocytes may have important clinical significance for protecting hepatic structure and function. It has been determined that serum deprivation triggers the accumulation of intracellular ROS and alteration of mitochondrial function, which leads to cell apoptosis (28). Serum deprivation is a commonly used method to induce cell apoptosis (29,30). In this study, human liver cells exhibited apparent apoptosis after serum deprivation, and with increased time in serum-free medium, the apoptotic effect became more severe. Moreover, in the early phase of serum deprivation, the mitochondrial membrane potential declined. Afterwards, cytochrome c was activated and released from the mitochondria into the cytoplasm, and the expression of caspase-3 was elevated. In conclusion, the serum deprivation-induced apoptosis of human liver cells occurs via the mitochondrial pathway.

Opioid receptors are widely expressed throughout the body, and it has been found that in addition to the central nervous system, a large number of opioid receptors are expressed in the liver. In the superfamily of opioid receptors, the δ-opioid receptor is mainly associated with the survival and proliferation of cells (31). Studies have shown that the δ-opioid receptor has protective functions in the liver. For example, δ-opioid receptors were found to have a protective role against liver damage in an animal model of cholestasis (32). In addition, endogenous opioid growth factor promotes the proliferation of liver cancer cells (10). These protective effects are attributed to the activation of the δ-opioid receptor on liver cell membranes. Our results showed that activation of the δ-opioid receptor reduces the apoptosis of human liver cells and down-regulates the expression of caspase-3, which constitutes a protective mechanism to sustain the physiological function of the liver.

The inhibitory function of the δ-opioid receptor against hepatic cell apoptosis is unknown, but its protective effect on myocardial cells is prominent. Yao et al (33) reported that activation of the δ-opioid receptor reduces the apoptosis of myocardial cells through inhibition of the ERK and IP3 pathways. Furthermore, Huang et al (34) reported that activation of the δ-opioid receptor inhibits apoptosis by promoting CGRP expression in myocardial cells. A cell-type specific discrepancy between liver cells and myocardial cells may exist. However, our findings are consistent with those of Wang et al (5), who found that activation of the δ-opioid receptor inhibited the serum deprivation-induced apoptosis of cardiomyocytes from a neonatal rat; the apoptotic appearance became more prominent when the concentration reached 1.0 μM. Further investigations are necessary to determine whether the activation of the δ-opioid receptor inhibits the apoptosis of other types of tissues and cells.

It has been confirmed that opioid receptors act through the PTX-sensitive G protein signal pathway (35) and the KATP signal pathway (36). In addition, Zhao et al (37) found that opioid receptors promote cell survival and proliferation via the ERK1/2 pathway. We determined that the activation of the δ-opioid receptor enhances PKC expression. PKC stimulation inhibits the apoptosis of different types of cells (38-40). Meanwhile, PKC also participates in the protection against hepatic ischemia reperfusion injury and is involved in the processes of cellular proliferation and apoptosis (19,41). We observed that despite δ-opioid receptor activation, the blockade of the PKC pathway increases the apoptosis of human liver cells. We conclude that the PKC pathway is a possible signal transduction pathway involved in δ-opioid receptor inhibition of human liver apoptosis induced by serum deprivation.

Our previous results demonstrated that serum deprivation induces the apoptosis of human liver cells through the mitochondrial pathway. These findings are similar to the research conducted by Li et al (42). It has been demonstrated that the δ-opioid receptor agonist DADLE protects nerve cells against apoptosis through the mitochondrial pathway (43), but it remains to be determined whether this anti-apoptotic mechanism is involved in our system. After activation of the δ-opioid receptor, the mitochondrial membrane potential rose markedly, the level of cytosolic cytochrome c decreased and the translocation of Bax from the cytoplasm to the mitochondria decreased, indicating that the protective effect of the δ-opioid receptor on liver cells is associated with the mitochondrial pathway.

In summary, we determined that serum deprivation-induced apoptosis of human liver cells occurs through the mitochondrial pathway. Activation of the δ-opioid receptor reverses the mitochondrial apoptotic pathway. Moreover, the protective function of the δ-opioid receptor on human liver cells is associated with PKC and the mitochondrial pathway. The elucidation of this anti-apoptotic mechanism is important for understanding the function of the δ-opioid receptor in human liver cell apoptosis and may lead to the development of therapeutics for human liver diseases.

Acknowledgements

We thank the Dalian Institute of Chemical Physics and the Chinese Academy of Sciences for the experimental supplies. This study was supported by National High Technology Research and Development Program (863 Program) funding (2006AA02A309).

References


