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Protein Kinase C mediates the effects of δ-opioid receptor stimulation on survival and apoptosis in neonatal cardiomyocytes cultured in serum-deprived condition

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The aims of the present study were to determine whether Delta opioid receptor (δ-OR) stimulation improved the survival of cardiomyocytes cultured in serum-deprived conditions, which impaired their growth. [D-Ala2, D-Leu5]-enkephalin (DADLE), a selective δ-OR agonist, at a concentration range of 0.1 μmol/L to 10 μmol/L for 48 h increased the viability of the cardiomyocyte under serum deprivation conditions. DADLE (0.1 μmol/L) also decreased the early cell apoptosis rate and the expression of Caspase-3. The effects of 0.1 μmol/L DADLE were abolished by 10 μmol · L^{-1} naltrindole, a selective δ-OR antagonist, or by blockade of protein kinase C (PKC) with its blockers, 10 μmol · L^{-1} GF109203X or 1 μmol/L staurosporine. Furthermore, 0.1 μmol · L^{-1} DADLE increased the expression of PKC, an effect abrogated by 10 μmol · L^{-1} naltrindole. The observations indicate that δ-OR stimulation improves the viability and reduces the apoptosis *via* PKC pathway in neonatal cardiomyocytes cultured in serum deprived conditions.

1. Introduction

Delta (δ) opioid peptides are produced by cardiomyocytes (Caffrey et al. 1994; Springhorn and Claycomb 1992) and δ-opioid receptors (OR) are present in the heart (Valtchanova-Matchouganska and Ojewole 2003), indicating that the δ-opioid peptides may play an important role in the regulation of the cardiac functions as paracrine or autocrine hormones. Previous studies in our laboratory have demonstrated that δ-OR stimulation enhanced the proliferation and development of cultured neonatal rat ventricular myocytes (Zao et al. 2008), indicating that the peptide was important in cell survival and development by direct action at the level of myocardium. It is also likely that δ-OR stimulation may play a crucial role in cell survival and development in conditions when the heart is exposed to an unfavorable environment.

Previous studies have identified that δ-OR stimulation has protective effects on the heart and cardiomyocytes towards the injuries. Morphine had very good protective effects against a high dose of DOX given as a multiple, low, unitary dose regimen *via* δ-OR stimulation (Kelishomi et al. 2008). Stimulation of cardiac delta-OR prevents irreversible cardiac cell damage in ischemia and reperfusion in *vitro*, and phosphorylation of Cx43 by PKC-epsilon plays a crucial role in this process (Miura et al. 2007). The delta-opioid agonist DADLE protects rabbit hearts at reperfusion through activation of the pro-survival kinases Akt and ERK (Maslov et al. 2006; Förster et al. 2007; Ikeda et al. 2006). Such experiments demonstrated the cardioprotective effect of δ-OR stimulation. However, there is no evidence for a protective effect of δ-OR stimulation for survival and development when the myocardiocytes are exposed to an unfavorable environment, such as serum-deprivation.

The protein kinase C (PKC) family of calcium and/or lipid-activated serine-threonine kinases is located downstream of nearly all membrane-associated signal transduction pathways (Molkentin and Dorn 2001). PKC is activated by δ-OR stimulation (Leblanc et al. 1998). A number of studies have implicated that PKC activation is associated with heart diseases such as heart failure and ischemic injury (Braz et al. 2002). Therefore, δ-OR stimulation may protect the heart in unfavorable conditions via activation of PKC.

In the present study we determined the effects of δ-OR stimulation on apoptosis and survival of neonatal cardiomyocytes under the condition of serum deprivation, which impairs the growth and development of the myocytes. We further determined the role of PKC by the use of its blockers and its expression upon δ-OR stimulation. Results showed that δ-OR stimulation with DADLE enhanced survival and reduced apoptosis of cultured myocardial cells after serum deprivation *via* the PKC signal transduction pathways.

2. Investigations and results

2.1. Effect of [D-Ala2,D-Leu5]-enkephalin (DADLE) on the viability of serum deprivation-cultured cardiomyocytes with and without blockade of δ-OR or PKC

After serum-deprived culture for 48 h, the A570, reflecting the viability, of the neonatal rat cardiomyocytes was mark-

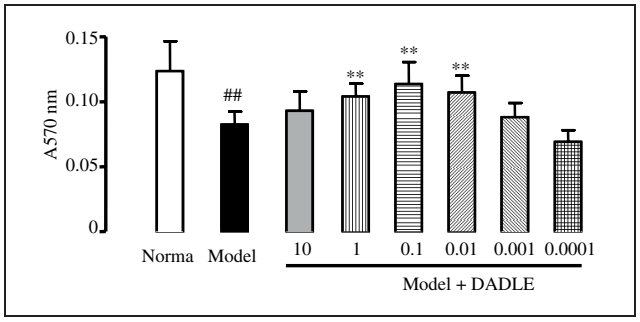

Fig. 1: Different concentration of [D-Ala^2,D-Leu^5]-enkephalin (DADLE) on cell viability of neonatal cardiac myocytes ($x \pm s$, $n = 8$). After the cells (1×10^5 cells per well) were cultured for 2 d, the medium was changed to DMEM and not supplemented with calf serum, 48 h later, DADLE was added to the medium and cultured for another 48 h. Cell viability was determined by crystal violet staining method. $^{**}P < 0.01$, compared with model

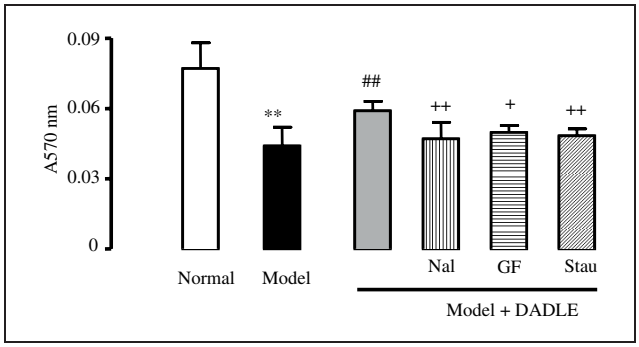

Fig. 2: Effects of [D-Ala^2,D-Leu^5]-enkephalin DADLE ($0.1\ \mu mol \cdot L^{-1}$) naltrindole ($10\ \mu mol \cdot L^{-1}$) GF109203X ($10\ \mu mol \cdot L^{-1}$) staurosporine ($1\ \mu mol \cdot L^{-1}$) on cell viability of neonatal cardiac myocytes ($x \pm s$, $n = 8$). After the cells (1×10^5 cells per well) were cultured for 2 d, the medium was changed to DMEM and not supplemented with calf serum, 48 h later, DADLE, Naltrindole GF109203X staurosporine added to the medium and cultured for another 48 h. Cell viability was determined by crystal violet staining method. $^{**}P < 0.01$, compared with normal; $^{\#\#}P < 0.01$, compared with model; $^{++}P < 0.01$ compared with DADLE

edly decreased. Continued culture in serum-free medium with DADLE at 0.01 μmol/L–10 μmol/L for 48 h increased the viability (Fig. 1). The increases were significant starting at 0.1 μmol/L and it seemed that a plateau was reached when the concentration reached 0.1 μmol/L. The effect of 0.1 μmol/L DADLE was abolished in the presence of 10 μmol/L naltrindole, a selective δ-OR antagonist (Fig. 2). The effect was also abolished by blockade of PKC with a PKC inhibitor, 10 μmol/L GF109203X, or 1 μmol/L staurosporine (Fig. 2).

2.2. Effect of [D-Ala2,D-Leu5]-enkephalin (DADLE) on the early apoptotic rate of serum deprivation-cultured cardiomyocytes with and without blockade of δ-OR or PKC

At 0.1 μmol/L DADLE also inhibited early apoptotic rate (Fig. 3). The inhibition was abolished in the presence of $10\ \mu mol \cdot L^{-1}$ naltrindole. The effect of DADLE at $0.1\ \mu mol \cdot L^{-1}$ was also significantly attenuated and abol-

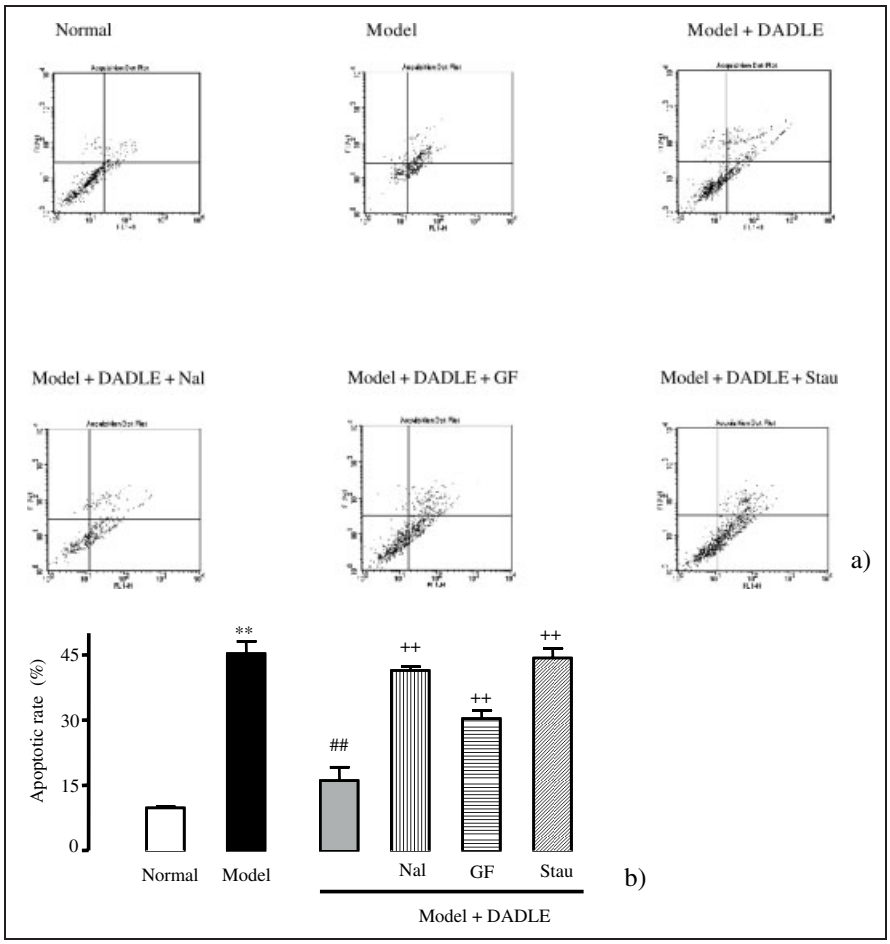

Fig. 3:
Apoptotic rate of flow cytometric diagram of AnnexinV-FITC/PI staining of the cardiomyocytes induced by serum deprivation ($\bar{x} \pm s$, $n = 3$). Method and time of cell culture were the same as in Fig. 1. DADLE, naltrindole, GF109203X and staurosporine were added to the medium at the same time and cultured for 48 h. (A) show the representative photographs. In each figure: LL (left lower): Living cells (AnnexinV-FITC-/P I-); LR (rightlower): apoptotic cells (AnnexinV-FITC+/PI-); UR (right upper): necroticcells (AnnexinV-FITC+/PI+). (B) show the apoptotic rate change. $^{**}P < 0.01$, compared with normal; $^{\#\#}P < 0.01$, compared with model $^{++}P < 0.01$ compared with DADLE

ished in the presence of GF109203X at 10 μmol · L^{-1} or staurosporine at 1 μmol · L^{-1}.

2.3. Effect of [D-Ala2,D-Leu5]-enkephalin (DADLE) on the G0/G1% ,S%, G2/M% phases DNA content in serum deprivation-cultured cardiomyocytes with and without blockade of δ-OR or PKC

Serum deprivation significantly decreased, while DADLE at 0.1 μmol · L^{-1} significantly increased, the G_0/G_1%, S%, G_2/M% phases DNA content (Fig. 4). The effect of DADLE was abolished by blockade of δ-OR with naltrindole at 10 μmol · L^{-1} and attenuated, respectively by blockade of PKC with either GF109203X at 10 μmol · L^{-1} or staurosporine at 1 μmol · L^{-1}.

2.4. Effect of [D-Ala2,D-Leu5]-enkephalin (DADLE) on the caspase-3 expression in serum deprivation-cultured cardiomyocytes with and without blockade of δ-OR or PKC

The caspase-3 expression was significantly increased following serum deprivation and DADLE at 0.1 μmol · L^{-1}

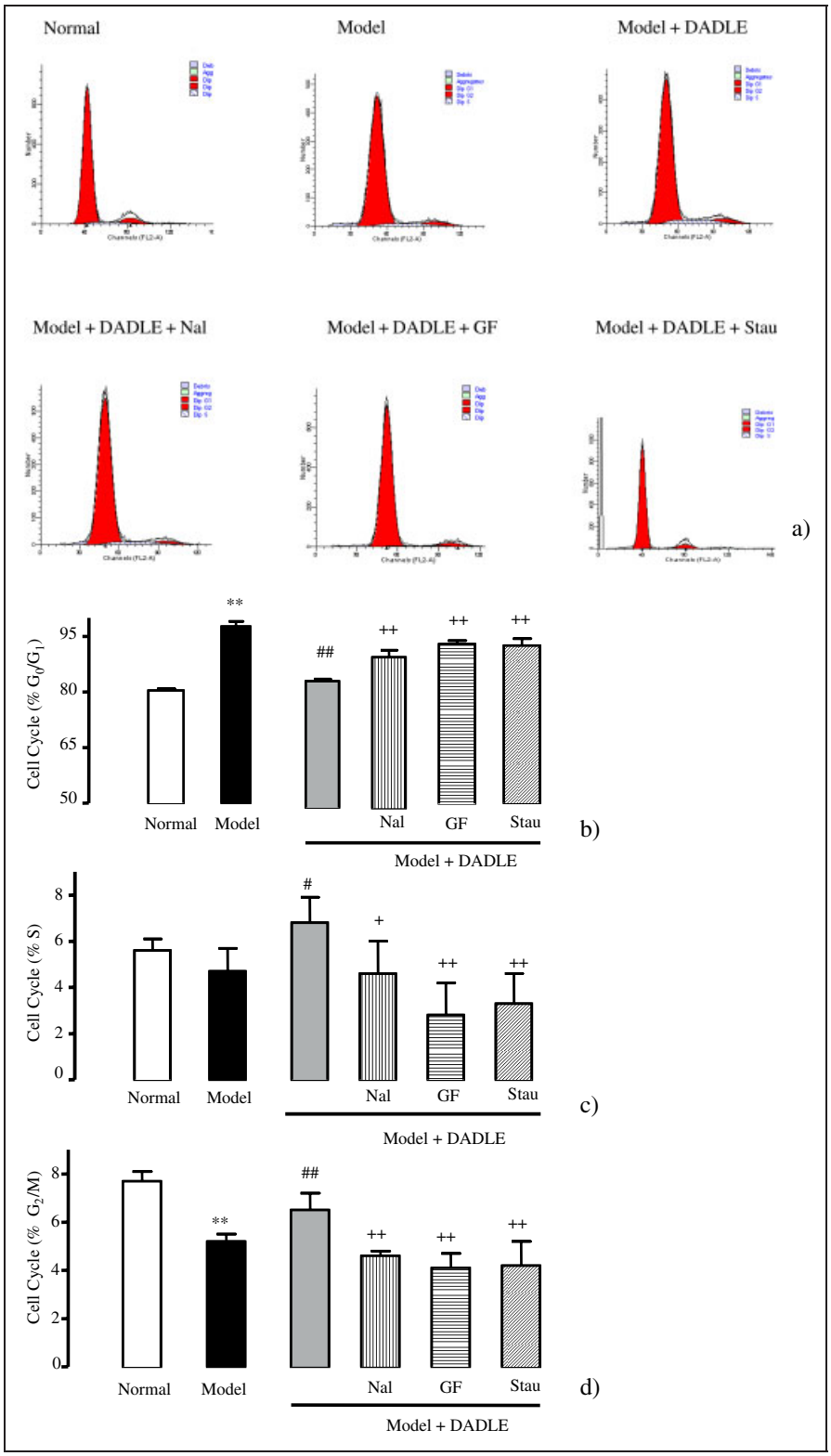

Fig. 4:
Effects of DADLE Naltrindole GF109203X and Staurosporine on cell cycle of cultured myocardial cells of neonatal rats ($\bar{x} \pm s$, $n = 3$). Method and time of cells culture were the same as in Fig. 1. DADLE, naltrindole, GF109203X and staurosporine were added to the medium at the same time and cultured for 48 h. (A) shown the representative photographs. (B) shown the ration of the cell cycle changes of G_0/G_1. (C) shown the ratio of cell cycle changes of S phase. (D) shown the ration of the cell cycle change of G_2/M. $^{**}P < 0.01$, compared with normal; $^{\#\#}P < 0.01$, compared with model; $^{\#}P < 0.05$, compared with model; $^{++}P < 0.01$ compared with DADLE; $^{+}P < 0.05$ compared with DADLE

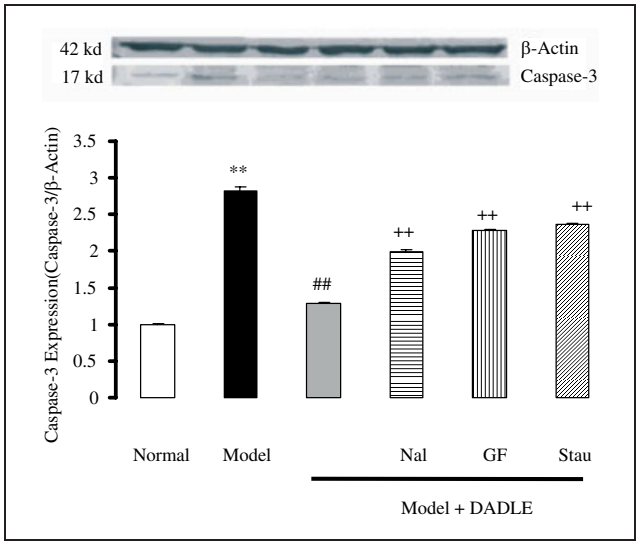

Fig. 5: Effects of different agents on caspase-3 expressed ($\bar{x} \pm s$, $n = 4$). Apart from the cells (1×10^6 per flask), method and time of cell culture were the same as in Fig. 1. DADLE, naltrindol, GF109203X and staurosporine were added to the medium at the same time and cultured for 48 h. 1 : normal; 2 : model; 3 : model + DADLE ($0.1\ \mu mol \cdot L^{-1}$;) 4 : model + DADLE ($0.1\ \mu mol \cdot L^{-1}$) + GF109203X ($10\ \mu mol \cdot L^{-1}$); 5 : model + DADLE ($0.1\ \mu mol \cdot L^{-1}$) + staurosporine ($1\ \mu mol \cdot L^{-1}$) $^{**}P < 0.01$, compared with normal; $^{\#\#}P < 0.01$, compared with model, $^{++}P < 0.01$ compared with DADLE

significantly decreased the caspase-3 expression (Fig. 5). The effect of DADLE was significantly attenuated in the presence of naltrindole at $10\ \mu mol \cdot L^{-1}$) or GF109203X at $10\ \mu mol \cdot L^{-1}$or staurosporine at $1\ \mu mol \cdot L^{-1}$.

2.5. Effect of [D-Ala2,D-Leu5]-enkephalin (DADLE) on the expression in of PKC in serum deprivation-cultured cardiomyocytes with and without blockade of δ-OR

PKC expression was significantly decreased following serum deprivation and 0.1 μmol/L DADLE significantly increased the PKC expression. The effect of DADLE was significantly attenuated in the presence of naltrindole at $10\ \mu mol \cdot L^{-1}$ (Fig. 6).

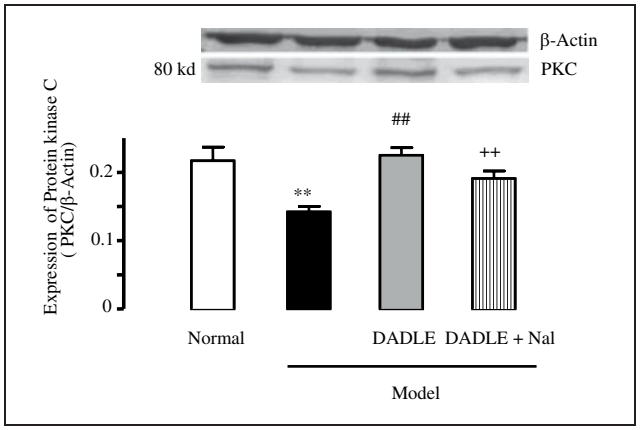

Fig. 6: Effects of different agents on protein kinase C (PKC) expressed ($\bar{x} \pm s$, $n = 4$). Apart from the cells (1×10^6 per flask), method and time of cell culture were the same as Fig. 1. DADLE, naltrindole, GF109203X and staurosporine were added to the medium at the same time and cultured for 48 h. 1 : normal; 2 : model; 3 : model + DADLE ($0.1\ \mu mol \cdot L^{-1}$;) 4 : model + DADLE ($0.1\ \mu mol \cdot L^{-1}$) + naltrindole; $^{**}P < 0.01$, compared with normal; $^{\#\#}P < 0.01$, compared with model; $^{++}P < 0.01$ compared with DADLE

3. Discussion

The first important observation of the present study was that DADLE, a selective δ-OR agonist, increased the cell viability, and reduced apopotic rate and expression of caspase-3, an apoptotic protein positively correlated to apoptosis index, in neonatal cardiomyocytes cultured in serum deprived conditions and the effects were abolished by blockade of δ-OR by its antagonist, naltrindole. The observations are evidence that δ-OR stimulation increases the viability and decreases cell death of neonatal cardiomyocytes in serum deprived conditions. A previous study in our lab has shown that a δ-OR stimulation increases growth of cardiomyocytes (Zao et al. 2008). So δ-OR stimulation enhances growth in both normal and unfavorable conditions.

The second important observation of the present study is that the effects of δ-OR stimulation with DADLE were not only abolished by blockade of δ-OR with its antagonist, naltrindole, but more importantly by blockade of PKC with its antagonist, GF109203X or staurosporin. In addition, DADLE enhanced the expression of PKC, an effect attenuated by naltrindole. The observations indicate that the effects of δ-OR stimulation involve the PKC signaling pathway. Previous studies have demonstrated a role of PKC in growth and development (Mayr et al. 2004). Protein kinase C-α has been shown to mediate proliferation and differentiation of chick myoblast induced by 1,25-dihydroxy-vitamin D3 (Capiati et al. 1999). In the present study, we only showed that blockade of PKC attenuated the effects of δ-OR stimulation on survival and apoptosis. Which PKC isoform is involved warrants further study.

Since 1996, many studies have demonstrated that a variety of cardiac pathological states including heart failure are associated with myocyte apoptosis in both humans (Narula et al. 1996; Olivetti et al. 1997) and animals (Teiger et al. 1996; Li et al. 1997). Apoptosis of cardiomyocytes is increased in human heart failure and several studies have subsequently suggested that cardiomyocyte apoptosis is a central mechanism in heart failure (Harsdorf 2004; Empel and de Windt 2004). Diverse signal transduction pathways, G proteins and protein kinases among them, likely contribute to heart failure, and the identification of essential control points have both fundamental and translational importance (Olson and Schneider 2003; Foo et al. 2005). The previous findings suggest a role for the activation of the apoptotic cascade in heart failure, which may involve the activation of proteolytic caspase-3 and cardiomyocyte loss (Narula et al. 2001; Narula et al. 1999). Activation of caspase-3 is followed by cardiomyocyte apoptosis, which is sufficient to induce cardiac failure (Wencker et al. 2003). Both apoptosis and necrosis may contribute to myocyte loss, the extent to which apoptosis plays a role in cardiovascular diseases is an open question.

We know that caspase-3 is a kind of apoptotic protein and caspase-3 precursor exits in cytoplasm in normal condition. Once caspase-3 is activated, cell apoptosis is inevitable. We used the Annexin V-FITC assay to determine myocyte cell surface changes that occur early in the apoptotic process by flow cytometry. The AnnexinV-FITC conjugate facilitates rapid fluorimetric detection of apoptotic cells, meanwhile, we have detected caspase-3 activated by Western blotting. Therefore, the expression of caspase-3 may play a significant role in apoptosis of myocardial cells by serum deprivation. We have demonstrated that δ-

opioid receptor stimulation promoted survival of neonatal cardiomyocytes, which increase survival index of cardiac myocytes, percentage of G_2/M in cell cycle and the expression of PKC, and decrease the apoptotic index of cardiac myocytes, percentage of G_0/G_1 in cell cycle and the expression activate caspase-3. The protective of DADLE was obviously blocked by δ-opioid receptor antagonist naltrindole at $10\,\mu mol \cdot L^{-1}$, the PKC inhibitor GF109203X at $10\,\mu mol \cdot L^{-1}$ and Staurosporine at $1\,\mu mol \cdot L^{-1}$, which decrease survival index of cardiac myocyte, percentage of G_2/M in cell cycle and the expression of PKC, increase apoptotic index of cardiac myocyte, percentage of G_0/G_1 in cell cycle and the expression of caspase-3. Based on the present results we think that the protective potential of DADLE in the failing heart may be due to one of the facts that DADLE activates the δ-opioid receptor, activates an opioid-like G protein-coupled receptor, promotes activation of PKC, and accordingly inhibits the expression of caspase-3. PKC may be an important mediator, which regulates dual modulation of cell survival and cell apoptosis in cultured neonatal rat cardiomyocytes. In conclusion, the present study has provided evidence that δ-OR stimulation enhances survival and reduces apoptosis under serum deprivation conditions, which involved the PKC signaling pathway.

4. Experimental

The experimental protocols were approved by Committee of Liaoning Medical College for the Use of Experimental Animals for Research and Teaching.

4.1. Culture of neonatal rat ventricular myocytes

Sprague-Dawley rats of 2 days of age (Animal Center of Liaoning Medical College), were killed and the heart was removed immediately. The ventricles were separated from the atrias, trisected and digested with 0.08% trypsin (Sigma, St Louis, MO, USA) at 37 °C. Ventricle myocytes were cultured as described previously (Wang et al. 2004; Shan et al. 2007). The digestion steps were repeated up to five times until the tissues were completely digested. The cells were combined, centrifuged at 10 min, 37 °C, 90 g and resuspended in chilled calf serum. To segregate myocytes from non-myocytes, a discontinuous gradient of 40.5 and 58.5% Percoll (Sigma) was prepared in balanced salt solution and ventricular cells were suspended in the 58.5% Percoll layer. After centrifugation at $270 \times g$ for 30 min, the upper layer consisted of a mixed population of non-myocyte cells and the lower layer consisted almost exclusively of myocytes. To purify myocardial cells, both layers (i.e. myocytes and non-myocytes) were washed twice by centrifugation (10 min, 37 °C, 90 g) and resuspension to remove all traces of Percoll (Zao et al. 2008). After the cells had been incubated twice on uncoated 10 cm culture dishes for 30 min to remove any remaining non-myocytes, approximately 5×10^5 or 1×10^6 /mL myocytes were plated and grown as confluent monolayers on 96-well plates (0.1 mL/well), 24-well cell plates (1 mL/well) or 25 cm^2 (bottom area) culture flasks (5 mL/flask) at 37 °C under an atmosphere of 5% CO_2, 95% air. The culture medium consisted of 15% heat-inactivated fetal bovine serum, 84% Dulbecco's Modified Eagle's Medium (DMEM) and 1% penicillin-streptomycin solution, known to enhance growth of cultured ventricular myocytes (Bilecki et al. 2000). After 48 h incubation, the wells or flasks were divided into test groups and the medium was replaced with free-serum DMEM supplemented with different agents for a further 48 h. Samples of the media were taken for assays.

4.2. Crystal violet staining assay for cell viability

Myocardial cells were cultured for 48 h in 96-well plates under controlled conditions (37 °C, 5% CO_2). Cell numbers were then assessed using the crystal violet staining technique (Zager 1999). In brief, after incubation was complete, cells were fixed by the addition of 5% glutaraldehyde, washed, dried and stained with crystal violet (0.1%; pH 6) for 20 min. Unfixed crystal violet was removed by washing four times and the plates were then dried. Finally, the adherent crystal violet in each well was solubilized with 0.2% Triton X-100 and the absorbance at 570 nm (A570) of each well was simultaneously determined by a DNM-9602G automated EIA Analyzer (DNM-9602G, Perlong Medicial Equipment CO, Beijing). The value for A570 was in direct proportion with cell number.

4.3. Flow cytometric analysis for identification and quantification of cell apoptosis

The method 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, Biosea Biotechnology Co, Beijing) and analyzed by flow cytometry to differentiate types of cell death. Cells of annexin-V-positive and PI-negative were classified as apoptosis or early-stage apoptosis cells. Briefly, cells were by digested 0.25% trypsin, washed three or four times and then stained 15–20 min until the cells were completely digested. After cells were digested, they were washed with PBS. Unfixed cells were stained by adding 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).

4.4. Analysis of cellular DNA content by flow cytometry

The cells were grown at 48 h confluence in 25 cm^2 (bottom area) culture flasks (5 mL/flask). Different agents were added to the medium at the same time and cultured serum-starved for 48 h. At the end of the treatment, the floating cells were collected by centrifugation and solution to produce a single cell suspension. The cells were then pelleted by centrifugation and washed twice with PBS. Then, the cell pellets were suspended in 0.5 mL PBS and fixed in 5 mL ice-cold 70% ethanol at 4 °C. After 1 h, the fixed cells were spun by centrifugation and the pellets were washed with PBS. After resuspension with 1 mL PI integration staining solution, the cells were incubated with RNase A (10 mg/L), PI (50 mg/L), 1% TritonX-100 and sodium citrate (1g/L) shaken for 30 min at 37 °C in the dark. The stained cells were analyzed using a FACScalibur flow cytometer.

4.5. Expression of caspase-3 by Western blot analysis

Western blot was performed with mouse monoclonal antibody against caspase-3 and the secondary antibody was goat anti-mouse mmunoglobulin G-horseradish peroxidase (IgG-HRP) conjugate. Cells were washed once with ice-cold PBS containing 100 mmol/L sodium orthovanadate and solubilized in lysis buffer (50 mmol/L Tris-HCl, 137 mmol/L NaCl, 10% glycerol, 100 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 10 mg/mL aprotinin, 10 mg/mL leupeptin, 1% Nonident P-40; pH 7.4). After centrifugation at $12000 \times g$ for 20 min, the supernatant was collected. Cells were dissolved in sample buffer containing 65 mmol/L Tris-HCl (pH 6.8), 3% SDS, 10% glycerol and 6 mol/L 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 thus obtained (60 mg) was separated, using 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transblotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The blots were incubated at 4 °C overnight with antibodies and the resulting bands detected using enhanced chemiluminescence. Antibodies to caspase-3 (1 : 1000 dilution; SantaCruz, CA) were used to detect the corresponding total protein levels. Intensities in resulting bands were quantified using an image-analysis system. The expression level of caspase-3 was corrected by β-actin. The results were shown in relative optical density.

4.6. Expression of PKC by Western blot analysis

Western blot was performed with mouse monoclonal antibody against PKC and secondary antibody was goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) conjugate. Cells were washed once with ice-cold PBS containing 100 mmol/L sodium orthovanadate and solubilized in lysis buffer (50 mmol/L Tris-HCl, 137 mmol/L NaCl, 10% glycerol, 100 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 10 mg/mL aprotinin, 10 mg/mL leupeptin, 1% Nonident P-40; pH 7.4). After centrifugation at $12000 \times g$ for 20 min, the supernatant was collected. Cells were dissolved in sample buffer containing 65 mmol/L Tris-HCl (pH 6.8), 3% SDS, 10% glycerol and 6 mol/L 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 thus obtained (60 mg) was separated using 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transblotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The blots were incubated at 4 °C overnight with antibodies and the resulting bands detected using enhanced chemiluminescence. Antibodies to PKC (1 : 1000 dilution; SantaCruz, CA) were used to detect the corresponding total protein levels. Intensities in resulting bands were quantified using an image-analysis system. The expression level of PKC was corrected by β-actin. The results were shown in relative optical density.

4.7. Statistical analysis

All data are expressed as mean ± S.E.M. For 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.

4.8. Materials

[D-Ala2, D-Leu5] enkephalin (DADLE), naltrindole, GF109203X, staurosporine, and DMEM were obtained from Sigma. Calf bovine serum was from Si Ji Qing Chemical (Hangzhou, China).

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