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Calcitonin gene related peptide (CGRP) inhibits norepinephrine induced apoptosis in cultured rat cardiomyocytes not via PKA or PKC pathways

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ABSTRACT

Evidence showed overrelease of norepinephrine can induce apoptosis in ventricle myocytes. Calcitonin gene related peptide and norepinephrine could be simultaneously up-regulated in early time of acute myocardial ischemia, suggesting a co-participation of calcitonin gene related peptide and norepinephrine in the pathology. In this study, we investigated a potential anti-apoptotic effect of calcitonin gene related peptide on myocardial apoptosis induced by norepinephrine and its link with the protein kinase A (PKA) or protein kinase C (PKC) pathway in cultured neonatal rat cardiomyocytes. Cultured cardiomyocytes were exposed to one of the treatments, separately: (1) 3 ml DMEM culture medium, (2) norepinephrine (10^{-5} mol/l), (3) H89 (3×10^{-5} mol/l), a specific PKA inhibitor, with norepinephrine (10^{-5} mol/l), (4) calcitonin gene related peptide at a range of concentrations (10^{-9} mol/l, 10^{-8} mol/l and 10^{-7} mol/l) with norepinephrine (10^{-5} mol/l) and (5) calcitonin gene related peptide (10^{-8} mol/l) with norepinephrine (10^{-5} mol/l) + $CGRP_{8-7}$ (10^{-7} mol/l), a specific antagonist of calcitonin gene related peptide receptor. Then, apoptosis rate and the activity of PKA and PKC were examined. The dose of norepinephrine induced a marked increase in apoptosis of the myocytes ($31 \pm 2\%$), compared to the control ($17 \pm 4\%$, $p < 0.05$). The pro-apoptotic effect of norepinephrine was attenuated by H89 (3×10^{-5} mol/l) or by calcitonin gene related peptide which could be completely reversed by $CGRP_{8-37}$. The activities of PKA and PKC were increased by norepinephrine but no difference in the activities of PKA and PKC was detected in the presence and absence of co-treatment with calcitonin gene related peptide (10^{-8} mol/l). Calcitonin gene related peptide inhibits norepinephrine induced apoptosis in cultured cardiomyocytes, which is mediated by CGRP receptor but unlikely to be mediated by PKA or PKC pathway.

Increased sympathetic nervous activity in the myocardium is a significant feature in patients with heart failure and acute myocardial ischemia and infarction [6,22,26]. Catecholamines are important regulators of myocardial contractility and metabolism [4,14,25,37]. However, excessive release or administration of catecholamines, including norepinephrine (NE), a main neurotransmitter of the sympathetic nerves, may result in myocardial injury [7,17,27]. Norepinephrine exerts its effect by binding to G-protein-coupled adrenergic receptors and then activating the cAMP-protein kinase A pathway in different tissues [3,8,14,39,31].

Primary sensory neurons conduct nociceptive information to the CNS, but also release neuropeptides, including calcitonin gene related peptide (CGRP), from peripheral nerve endings when stimulated [11,35,36]. CGRP, a principal transmitter in sensory nerves, is widely distributed in the cardiovascular system [18,20,21,28]. Previous investigations have demonstrated that CGRP plays an important protective role in the ischemic and reperfusion injury of the myocardium, brain, gastrointestinal system, and other tissues [4,35,5,12,13,15,23,28,29,33,34]. Evidence indicates that protein kinase A (PKA) or protein kinase C (PKC) pathways may mediate the effects of CGRP [8,2,19,30,32]. However, the protective effect of CGRP has not been studied on apoptosis of myocytes induced by norepinephrine. In the present study, we investigated a potential protective effect of CGRP on NE-induced apoptosis and the association of the anti-apoptotic effect with PKA or PKC mechanisms, in cultured cardiomyocytes of neonatal rats.

Myocyte cultures were prepared as reported [37,17,14]. Hearts from 1 to 2 day old Sprague–Dawley rats (Shanxi Medical University Experimental Animal Laboratory) were removed and the ventricles were dissected out and minced and then digested in 2 ml of collagenase (1 mg/ml, Type II, Invitrogen Corporation, California, USA) in phosphate buffer (PBS) for 3 min at room temperature.

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The free-floating cardiomyocytes were centrifuged in cold culture medium containing fetal bovine serum at 800 rpm for 3 min. The cells were collected and re-suspended in 12 ml DMEM (Dulbecco's modified eagle medium) in a 90 mm culture dish, and then incubated for 60 min at 37 °C in a carbon dioxide incubator (Thermo, USA) with a gas phase of 5% CO_2 in humidified air. The floating cardiomyocytes were collected and cell numbers were adjusted to 5×10^5 cells/ml in DMEM supplemented with 10% fetal bovine serum and 0.1 μmol/l 5-bromo-2′-deoxyuridine (Sigma–Aldrich, MO, USA) to prevent non-myocardial mesenchymal cells (NMCs) proliferation. 3 ml of the cell suspension were added into each well of a six-well cell culture cluster (Corning Gilbert Inc., AZ, USA), with cover slips (pretreated with poly-lysine) for TUNEL assay and without cover slips for PKA or PKC assay. After 48 h of incubation, more than 70% of the cells adhered to the culture dish or cover slips. Thereafter, the cultures were re-fed daily with fresh DMEM culture medium and ready for the experiments.

Serum-free DMEM culture medium (3 ml) was exposed to each well of the 6-well cell culture cluster. After 1 h incubation, the cardiomyocytes were exposed to one of the test agents or the combination of agents for 15 min: (1) control vehicle solution, (2) norepinephrine (10^{-5} mol/l), (3) norepinephrine (10^{-5} mol/l) + CGRP (at 10^{-9} mol/l, 10^{-8} mol/l and 10^{-7} mol/l), (4) norepinephrine (10^{-5} mol/l) + a PKA inhibitor, H89 (3×10^{-6} mol/l) and (5) norepinephrine (10^{-5} mol/l) + CGRP (10^{-8} mol/l) + $CGRP_{8-37}$ (10^{-7} mol/l), a specific antagonist of CGRP receptor. The concentrations of norepinephrine and CGRP were selected according to our pilot experiments [38]. 15 min after the exposure to the agent or agents, apoptosis of the myocytes and the activity of PKA and PKC were examined, respectively.

The TUNEL assay was performed with a Roche in situ cell death detection kit (Roche, Switzerland) according to the manufacturer's instructions. Briefly, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 60 min. Washed with PBS, then the cells were incubated in penetrating solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. After 60 min incubation with the TUNEL reaction mixture (at 37 °C), the cells were covered with converter-POD for 30 min at 37 °C in a humidified chamber and then rinsed with PBS. DAB (3,3N-diaminobenzidine tertrahydrochloride) was added for about 1 min and then the cells were counterstained with hematoxylin. The cells were dehydrated, mounted and analyzed under light microscope. The following findings were considered to represent apoptosis [16]: (a) marked condensation of chromatin and cytoplasm (apoptotic cells); (b) cytoplasmic fragments with or without condensed chromatin (apoptotic bodies); and (c) intra- and extracellular chromatin fragments (micronuclei). The apoptosis of the cells was examined by counting cells in 10 randomly chosen fields (40×). The rate of apoptotic myocytes was calculated using the formula:

$$\mathrm{RA} = \frac{N_\mathrm{p}}{N_\mathrm{t}} \times 100\%$$

In the formula, RA stands for rate of apoptosis; N_{pA}, the number of positively stained apoptotic myocytes; N_t, the total number of myocytes counted.

The PKA activity was determined according to the report by Goueli [10]. The cultured myocytes were rinsed with PBS and scraped from the culture cluster with a plastic lifter (Corning Incorporated, New York, USA). Then 100 μl of cold PKA extraction buffer containing: [25 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 ml 100 mmol/l PMSF (phenylmethanesulfonyl fluoride) in 100% ethanol] were added and the cells were incubated for 30 min on ice. The lysate from cell extracts was centrifuged at 14,000 × *g* for 5 min at 4 °C. The concentration of total protein was normalized in each group. The supernatant was assayed for PKA activity using the PepTag non-radioactive PKA assay kit (Promega, Madison, WI, USA) as described in the Promega Technical Bulletin. The assay was based on the changes in the net charge of the fluorescent PKA substrates (L-R-R-A-S-L-G), phosphorylated and non-phosphorylated. The phosphorylated substrate migrated toward the positive electrode, whereas the non-phosphorylated migrated toward the negative electrode, when electrophoresed in 0.8% agarose gel (100 V, for 20 min). The gel was photographed on an ultraviolet transmittance system (BIO-RAD Molecular Imager Gel DOC XR System), and analyzed using Quantity One software (BIO-RAD, version 4.6.7, USA).

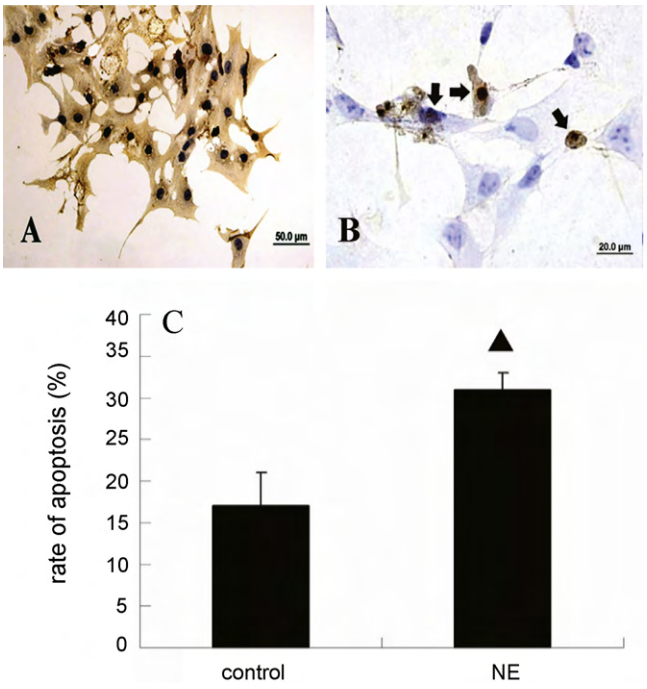

Fig. 1. Apoptosis in cultured myocytes: (A) over 98% of the cultured cells were characterized as α-actin. (B) Apoptosis of the cultured cardiomyocytes (arrows show the apoptotic cells). The bar = 50 μm in (A) and 20 μm in (B). (C) Summary of the rates of apoptosis in the cultured myocytes treated the norepinephrine (NE) and the vehicle solution (control). ▲$p < 0.05$ compared with the control.

Data were expressed as means ± standard deviation (SD). Comparison between the means was analyzed with one-way ANOVA test followed by Bonferroni post hoc test. In all tests, statistical significance was accepted at the level $p < 0.05$.

Over 98% of the cells were characterized as α-actin positive, which was defined as cardiomyocytes (Fig. 1A). The apoptosis rate in norepinephrine group was significantly increased (31 ± 2%) than that of the control group (17 ± 4%, $p < 0.05$, Fig. 1C). There was a significant decrease in the apoptosis of the cells when CGRP (at 10^{-8} mol/l and 10^{-7} mol/l) was added with NE (Fig. 2). The anti-apoptotic effect of CGRP (10^{-8} mol/l) could be completely reversed by $CGRP_{8-37}$, a specific CGRP receptor (Fig. 3), resulting in a rate of apoptosis of 32 ± 4%, indicating the anti-apoptotic effect of CGRP was mediated by specific CGRP receptor. The PKA inhibitor, H89 (at 3×10^{-5} mol/l), could also cause anti-apoptotic effect on NE-induced apoptosis (Fig. 3).

Compared with the control group, the activities of PKA were increased when NE (10^{-5} mol/l) alone or NE (10^{-5} mol/l) + CGRP (10^{-8} mol/l) was administrated. However the PKA activity was significantly reduced when NE (10^{-5} mol/l) was given with H89 ($p < 0.05$, Fig. 4). No difference in the PKA activity was detected between the NE and NE + CGRP (10^{-8} mol/l) groups ($p > 0.05$, Fig. 4). $CGRP_{8-37}$ (10^{-7} mol/l) significantly reduced PKA activity when given with NE (10^{-5} mol/l) and CGRP (10^{-8} mol/l).