Calcitonin Gene-Related Peptide Inhibits Cardiomyocyte Injury Induced by Hypoxia/Reoxygenation via Restoration of Cytosolic and Mitochondrial Calcium

Mu-Rong Li¹, Zheng Guo¹²³ and Lu Chen¹

ABSTRACT

Background: Calcium overload plays an important role in myocardial ischemia and reperfusion injury. Calcitonin gene-related peptide (CGRP) presents a property of cardioprotection, underlying which the mechanism is still elusive. In this study, we investigated the association of CGRP in attenuation of myocyte injury induced by hypoxia/reoxygenation (H/R) with modulation of cytosolic and mitochondrial calcium.

Methods: Cultured cardiomyocytes were assigned to four groups, 1) control group, without any treatment with any test agent, 2) H/R group, cells exposed to H/R, 3) H/R + CGRP group, cells treated with CGRP (at 10⁻⁸ mol/L) at the beginning of reoxygenation, and 4) H/R + CGRP + CGRP₈⁻₃⁷ group, cells treated with CGRP (10⁻⁸ mol/l) and CGRP₈⁻₃⁷, a special antagonist of CGRP receptor (at 10⁻⁷ mol/l), 1 hour before start of the hypoxia. The changes in calcium concentration, cell apoptosis, lactate dehydrogenase (LDH) and caspase-3 were analyzed using immunocytochemical, terminal deoxyuridine triphosphate (dUTP) deoxynucleotidyltransferase nick end-labeling (TUNEL) staining and enzyme-linked immunosorbent assay (ELISA) respectively.

Results: The greater levels of the apoptosis ratio, LDH, caspase-3, the cytosolic and mitochondrial calcium in H/R group were detected (all P< 0.05), compared to those in the control group, respectively. CGRP significantly attenuated the increases of the LDH, caspase-3 and myocyte apoptosis, with reduction of cytosolic and mitochondrial calcium in the myocytes treated with H/R. The effect of CGRP was reversed by CGRP₈⁻₃⁷.

Conclusions: The findings in this study suggested that CGRP prevents myocyte injury induced by H/R in cultured cardiomyocytes, which may be associated with homeostasis of cytosolic and mitochondrial calcium.

Background: ischemia/reperfusion injury (IRI) is defined as the damage to cardiac tissue when blood supply returns after a period of ischemia (1). Among the therapeutic strategies for IRI, ischemic preconditioning and post-conditioning (2, 3) are effective in promotion of survival of myocytes (4), via activation of intrinsic cardioprotective mechanisms, in which many factors and molecules participate (2, 5). Calcitonin gene-related peptide (CGRP), a principle transmitter of capsaicin-sensitive sensory nerves, plays an important role in the mediation of the cardioprotection afforded by ischemic pre- and post-conditions (6). However, the precise mechanism underlying the cardioprotective effects of CGRP is still unclear. Overload of cytosolic and mitochondrial calcium was found to play a pivotal role in the IRI (7, 8), which is a consequence of the insults by pro-injury...
factors (9). Previous reports from other and our
groups demonstrated a dose-dependent inhibitory
effects of CGRP on inward calcium currents
in isolated myocytes (10, 11). However, whether
there is an association between CGRP pro-
duced cardioprotection and modulating the cal-
cium inside the myocytes is still unknown.

In this study, we tested the hypothesis that
the fact CGRP protects the cardiomyocyte from
injury induced by H/R is associated with modu-
lating the cytosolic and mitochondrial calcium,
using cultured neonatal myocytes of rats. The
setting facilitates the clarification of a direct ac-
tivity of CGRP on the myocytes.

MATERIALS AND METHODS

Ethics Statement

The study was reviewed and approved by the In-
stitutional Animal Care and Use Committee of
Shanxi Medical University and conducted in ac-
cordance with the guidelines for the care and
use of laboratory animals (National Institute of
Health Guide for the Care and Use of Laborato-
ry Animals, NIH Publications No. 80- 23, re-
olaw.htm).

Cardiomyocyte Culture and Induction of Cell
Injury

Myocyte cultures were prepared and the cardio-
myocytes were identified as we previously report-
ed (12). Briefly, the hearts were collected from
the neonates of Sprague- Dawley rats (Shanxi
Medical University Experimental Animal Labora-
tory), within 72 hours of birth, after euthanasia
with ether. Then the tissues of the ventricles
were minced and digested in 2 ml of solution
containing collagenase type II (1 mg/ml, Invitro-
gen Corporation, Carlsbad, CA, USA) for 3- 5
minutes at 37°C. The cardiomyocytes were centrifuged at 1000 rpm for 5 minutes. The cells were collected and re-suspended in 12 ml Dulbecco’s modified eagle medium (DMEM) in a 90 mm culture dish, and then incubated for 60 minutes at 37°C in a carbon dioxide (CO₂) incubator (Ther-
mo, Hudson, NH, USA) with a gas phase of 5% CO₂ in humidified air. The floating cardiomyo-
cytes were collected and cell numbers were ad-
tioned to 5 × 10⁷ cells/ml in DMEM supplemen-
ed with 10% fetal bovine serum and 0.1 μmol/L
of 5-bromo-2'- deoxyuridine (Sigma-Aldrich, St.
Louis, MO, USA) to prevent non-myocardial
cells proliferation. Then, 3 ml of the cell suspension were added into each well of the six-well
cell culture clusters (Corning Gilbert Inc., Glen-
dale, AZ, USA). For analysis of cell apoptosis
with terminal deoxyuridine triphosphate (dUTP)
deoxynucleotidyltransferase nick end-labeling
(TUNEL) staining and the mitochondrion and cy-
tosolic calcium assay, the myocytes were cultured
on cover slips (pretreated with polylysine).

After 72 hours of incubation, the hypoxia/re-
oxxygenation (H/R) of the cultured myocytes was
conducted as follows. The cell culture clusters
were transferred into a hermetic chamber (5,
000 cm³) with circulating nitrogen (99.9% of N₂)
at rate of circulation of 0.5 l/minute, at
37°C. The culture medium was replaced by the
solution containing NaHCO₃ (6.0 mmol/l), Na-
Cl (98.5 mmol/l), KCl (10.0 mmol/l), CaCl₂ (1.0
mmol/l), sodium lactate (40 mmol/l), MgSO₄
(1.2 mmol/l) and HEPES (20 mmol/l) (13),
which had been saturated with 99.9% of N₂ for
2 hours (at a rate of 0.5 l/minute). After 3 hours
of incubation in the oxygen-deprived culture
(partial pressure of oxygen [PO₂] <10 mm Hg),
the medium was replaced by standard DMEM
(PO₂ >100 mm Hg) and the cells were incubated
in it for 2 hours.

Experimental Protocol

As shown by figure 1, the cultured cells were
randomly divided into four groups. The control
group, the cells were kept in normoxic culture
for 5 hours, without any treatment. The H/R
group, the cells were treated with the hypoxia
for 3 hours followed by the reoxygenation for 2
hours, without any other treatment. The CGRP
postconditioning group (H/R + CGRP), the cells
were treated with the H/R and CGRP, given at
10⁻⁸ mol/L (the final concentration, Tocris, UK)
immediately before the start of the reoxygena-
tion. The CGRP₆-₃₇ antagonizing group (H/R +
CGRP₆-₃₇ + CGRP), CGRP₆-₃₇ (Tocris, UK), a spe-
cific antagonist of CGRP receptor, was adminis-
trated (at the final concentration of 10⁻⁷ mol/L),
1 hour before the induction of cell-hypoxia,
then the treatments of H/R and CGRP (10⁻⁸ mol/
L) were followed as scheduled. The doses of
CGRP and CGRP_{8-37} used in this study were determined according to our previous studies (11, 14). The cell injury was evaluated by examinations of the cell apoptosis and the alterations of lactate dehydrogenase (LDH) and caspase-3.

**Determination of Cardiomyocyte Apoptosis**

The TUNEL assay was performed to analyze the cell apoptosis as previously reported (11, 14). Briefly, at the end of the reoxygenation, the cells were fixed with 4% paraformaldehyde for 1 hour at room temperature and then the cells were processed as the instruction for TUNEL assay. The apoptotic cells were visualized by stain with an in situ cell death detection kit (Roche, Switzerland). Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI) and visualized by microscopy. Apoptotic index was calculated as the percentage of apoptotic cells and total number of cells.

**Measurements of LDH and Caspase-3**

The changes of LDH and caspase-3 were determined by enzyme-linked immunosorbent assay (ELISA), as we previously reported (15, 16). Briefly, at the end of the experiments, 100 μl of culture medium collected from each well for the assessment of LDH and then the cultured myocytes in each well were rinsed with phosphate-buffered saline (PBS) and scraped from the culture cluster with a cell lifter (Corning Gilbt Inc., Glendale, AZ, USA), then loaded into a 1.5 ml EP tube. Then 100 μl of cold lysis buffer were added and the cells were incubated for 60 minutes on ice. The lysate from cell extracts was centrifuged at 14,000 × g for 10 minutes at 4°C. The supernatant was collected. The bicinchoninic acid assay (BCA) method was used to determine the protein content extracted from the cultured cells and the culture medium collected from each well, respectively, with Pierce BCA Protein Assay Kit (Thermo Scientific, Hudson, NH, USA). Then LDH and caspase-3 were determined using a rat LDH kit (ScienCell, Carlsbad, CA, USA) and a rat caspase-3 kit (R&D Systems Inc., Minneapolis, MN, USA) respectively. The results were produced by using a microplate reader (Thermo Multiskan Ascent microplate spectrophotometer, Thermo electron corporation, USA) and expressed as fold of control.

**Determination of Cytosolic and Mitochondrial Calcium**

Cytosolic and mitochondrial calcium was analyzed by co-incubating the cells with a cell-permeant but mitochondria- impermeant calcium fluorophore, Fluo-3 AM (5 μmol/l, Beyotime, China) and a mitochondria-permeant calcium fluorophore, Rhod-2 AM (5 μmol/l, Sigma-Aldrich, St. Louis, MO, USA), respectively, as reported (17). Cells were visualized by microscopy and photographed. Quantitative analysis of the images was carried out using ImageJ, a NIH software. The calcium concentration was expressed as proportion of the fluorescence intensity (FI).

**Immunofluorescence of CGRP Receptor**

After 72 hours of incubation, CGRP receptor was detected using immunofluorescence assay with antibodies against calcitonin receptor-like receptor (CRLR, 1:50, Santa Cruz, Dallas TX, USA) and receptor-activity-modifying proteins 1 (RAMP-1), 1:50, Santa Cruz, USA). The cells were divided into two groups (N=3), dyed with CRLR antibody and RAMP-1 antibody respectively. Cells were visualized by microscopy and photographed.

**Statistical Analysis**

Data were expressed as means ± standard deviation (SD). Statistical analysis of data was performed using ANOVA with post hoc Tukey’s test when multiple comparisons were made, and Student’s t-test for comparisons of two groups. Differences were considered statistically significant at \( p < 0.05 \).
formed by one-way analysis of variance (ANOVA) and by the Tukey’s test. A value of $P<0.05$ was considered to be statistically significant. All data analysis was conducted with the SPSS 13.0 software package.

**RESULTS**

**Cardiomyocyte Apoptosis**

As shown in figure 2, a significant increase of apoptotic ratio of the myocytes was detected in H/R group, by 80%, compared to the control group ($P<0.001$). Treatment with CGRP significantly attenuated the cell apoptosis in the H/R group, by 36%, when compared to that in the H/R group ($P<0.001$). CGRP$_{8-37}$, a specific antagonist of CGRP receptor, effectively reversed the effect of CGRP, by 85% ($P=0.001$).

**Caspase-3 and LDH**

As the apoptosis ratio, greater levels of caspase-3 and LDH were detected in H/R group, when compared to those of controls (caspase-3: $28.85 \pm 0.60 \mu\text{mol/g}$ vs $19.13 \pm 1.61 \mu\text{mol/g}$; and LDH: $20.23 \pm 0.92 \text{U/g}$ vs $11.58 \pm 0.39 \text{U/g}$, all $P<0.001$, Figure 3A, 3B). CGRP significantly attenuated the elevations of caspase-3 and LDH, compared to the values obtained from H/R group (caspase-3: $21.54 \pm 0.7 \mu\text{mol/g}$ vs $28.85 \pm 0.60 \mu\text{mol/g}$; LDH: $13.74 \pm 0.82 \text{U/g}$ vs $20.23 \pm 0.92 \text{U/g}$, all $P<0.001$; Figure 3B). The effects of CGRP were partially reversed by CGRP$_{8-37}$ (caspase-3: $24.98 \pm 0.50 \mu\text{mol/g}$ vs $21.54 \pm 0.71 \mu\text{mol/g}$, $P=0.01$ and $24.98 \pm 0.50 \mu\text{mol/g}$ vs $28.85 \pm 0.60 \mu\text{mol/g}$, $P=0.005$; LDH: $16.99 \pm 0.44 \text{U/g}$ vs $13.74 \pm 0.82 \text{U/g}$, $P=0.002$ and $16.99 \pm 0.44 \text{U/g}$ vs $20.23 \pm 0.92 \text{U/g}$, $P=0.002$), indicating the specificity of the actions of CGRP.

**Cytosolic and Mitochondrial Calcium**

As shown in figure 4, significantly higher concentrations of cytosolic and mitochondrial calcium were observed from the myocytes in H/R group, compared to those of controls (cytosolic, $P=0.006$; mitochondrial, $P=0.003$). Exogenously administrated CGRP (at $10^{-8} \text{mol/l}$) significantly inhibited both of the increases of the concentrations of the cytosolic and mitochondrial calcium (cytosolic, $P=0.006$; mitochondrial, $P=0.034$). The effects of CGRP on calcium were blocked by CGRP$_{8-37}$, indicating the effects mediated by the specific receptor of CGRP (cytosolic, $P=0.014$; mitochondrial, $P=0.043$).

**CGRP Receptors**

As shown in figure 5, the green fluorescence showed CRLR (Figure 5A) and RAMP-1 (Figure 5B), respectively. CGRP receptors in the cardiomyocytes were distributed in the cytoplasm and nucleus.

**DISCUSSION**

The important findings of this study were the cardioprotective effects of CGRP and its association with homeostasis of cytosolic and mitochondrial calcium. Although CGRP has shown cardioprotective properties, reduced infarct size (16, 18) and improved cardiac performance (19), few underlying mechanisms are known. Previous reports from other and our groups demonstrated that CGRP attenuated the increases in L-type calcium current of cardiomyocytes (10, 11) and apoptosis of cultured cardiomyocytes (12, 14), arising a potential effect of the peptide on calcium modulation. Here in this study, we tested the hypothesis that CGRP may
protect cardiomyocytes from the injury induced by hypoxia and reoxygenation, which may be associated with modulation of cytosolic and mitochondrial calcium. Firstly, the experimental setting of H/R in this study induced the significant and reproductive cell injury in the cultured cardiomyocytes, shown as increase of LDH, caspase-3 and cell apoptosis. The experimental setting partially mimicked the environment of cardiomyocytes in acute ischemia and reperfusion, which is characterized by lower PO₂ followed by reoxygenation and the adaptive responses of the cardiomyocytes to the changes of oxygen in the surrounding matrix, but without neural and paracrine humoral modulations. Secondly, the distribution of CGRP receptors were localized in the myocytes, which is an important base on which the pharmacological experiments with CGRP and CGRP₈-₃₇ can be possibly carried out in this study. This experimental model was applicable to test the protective effect of CGRP on cardiomyocytes.

Apoptosis is closely associated with many cardiovascular diseases (20). Caspase-3 stands at the junction of pathways mediated by other caspases in the cell, activation of which results in an increase of cell apoptosis, an enlargement of the infarct size and impairment of cardiac functions (21, 22). Increase of the amount of caspase-3 observed in this study served as an indicator of activation of the pro-apoptotic pathways by the H/R, as it went up well with the increases of the myocyte apoptosis, which also paralleled well with the increases of cytosolic and mitochondrial calcium, presenting an association of the up-grading of the calcium concentration with the activation of caspase-3 and increase in apoptosis of the cardiomyocytes. Furthermore, down-regulating the calcium concentrations by CGRP, in this study, went well with the reduction of caspase-3 and an attenuation of apoptosis of the cardiomyocytes, clearly presenting the association of homeostasis of cytosolic and mitochondrial calcium with increase of cell survival. The findings may suggest that the cardioprotective effect of CGRP may be associated with securing the homeostasis of cytosolic and mitochondrial calcium.

The release of LDH to the surrounding medium is a reliable marker of myocyte injury that re-
lates to cell death (23, 24). Therefore, increased level of LDH by H/R and inhibition of the elevation of LDH by CGRP served as one of the supporting quantitative parameters presenting the cell injury and the effect on cell protection from the injury, respectively, in this study. The results, obtained in this study on the alteration of apoptosis and LDH with the changes in caspase-3 and the calcium, without and with CGRP, clearly demonstrated a cardioprotective effect of CGRP and its association with modulation of the cytosolic and mitochondrial calcium.

The calcium signaling system is highly complex and intimately related with excitation-contraction coupling mechanism, gene expression, enzyme functions and cardiomyocyte injury (25, 26). Myocardial ischemia and reperfusion induces increase of calcium in the cardiomyocytes (25, 26), which is responsible for cell death and posts ischemic contractile dysfunction (27, 28). An increase in cytosolic calcium will stimulate calcium-dependent phospholipases, inducing the breakdown of cell membranes and release of toxic productions (25). It is well known, that calcium overload may disturb the cell metabolism, via causing dysfunction of mitochondria (29), which plays a crucial role in cell apoptosis (25), by an interaction of the endoplasmic reticulum and the mitochondria, upon pro-apoptotic stimulus, and induces the release of calcium from endoplasmic reticulum and calcium overload in mitochondria (30). Mitochondrial uptake of calcium is a crucial process in cell physiology (19, 31). Overload of mitochondrial calcium induces the opening of the MPTP (8, 32), which may lead to the release of cytochrome C and other pro-apoptotic molecules that initiate the apoptotic cascade (32). Elevation of the levels of calcium also increases calcium-ATPase activity and results in enhancement of consumption of ATP, which may lead to cell death (25) and alleviation of cytosolic and mitochondrial calcium overload is an effective way to facilitate cell survival (25, 26). The effects of CGRP observed in this study were similar to the effects of diltiazem, a calcium blocker, on calcium elevation and cell apoptosis.

The findings of this study expended current knowledge on the mechanism of CGRP on cardioprotection. However, the authors of this article would state that there were no groups of calcium ionophore and calcium antagonist. We would further investigate and elucidate the cellular and molecular mechanisms of cardioprotection induced by CGRP and its relationship with calcium modulation.

CONCLUSION

It could be concluded that CGRP produces cardioprotection, which may be associated with securing homeostasis of cytosolic and mitochondrial calcium.

This work was supported by grants from the National Natural Science Foundation of China (30972860 to Zheng Guo).

All authors have no other financial support and potential conflicts of interest for this work.

Zheng Guo conceived, designed, navigated the study and wrote the manuscript critically for important intellectual content. Lu Chen and Mu-Rong Li carried out the experiments, performed the data analysis. Mu-Rong Li drafted the manuscript.

References