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Molecular mechanisms underlying oxytocin-induced cardiomyocyte protection from simulated ischemia–reperfusion

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ABSTRACT

Oxytocin (OT) stimulates cardioprotection. Here we investigated heart-derived H9c2 cells in simulated ischemia–reperfusion (I–R) experiments in order to examine the mechanism of OT protection. I–R was induced in an anoxic chamber for 2 hours and followed by 2 h of reperfusion. In comparison to normoxia, I–R resulted in decrease of formazan production by H9c2 cells to 63.5 ± 1.7% (MTT assay) and in enhanced apoptosis from 1.7 ± 0.3% to 2.8 ± 0.4% (Tunel test). Using these assays it was observed that treatment with OT (1–500 nM) exerted significant protection during I–R, especially when OT was added at the time of ischemia or reperfusion. Using the CM-H_2DCFDA probe we found that OT triggers a short-lived burst in reactive oxygen species (ROS) production in cells but reduces ROS production evoked by I–R. In cells treated with OT, Western-blot revealed the phosphorylation of Akt (Thr 308, p-Akt), eNOS and ERK 1/2. Microscopy showed translocation of p-Akt and eNOS into the nuclear and perinuclear area and NO production in cells treated with OT. The OT-induced protection against I–R was abrogated by an OT antagonist, the Pi3K inhibitor Wortmannin, the cGMP-dependent protein kinase (PKG) inhibitor, KT5823, as well as soluble guanylate cyclase (GC) inhibitor, ODQ, and particulate GC antagonist, A71915. In conditions of I–R, the cells with siRNA-mediated reduction in OT receptor (OTR) expression responded to OT treatment by enhanced apoptosis.

In conclusion, the OTR protected H9c2 cells against I–R, especially if activated at the onset of ischemia or reperfusion. The OTR-transduced signals include pro-survival kinases, such as Akt and PKG.

1. Introduction

Until recently oxytocin (OT) had been considered a neurohypophyseal hormone acting primarily in reproduction, brain neuromodulation and central regulation of blood pressure. Our work has revealed a functional OT system in the human and rat heart and uncovered additional roles for this hormone (Gutkowska and Jankowski, 2012). The accumulated evidence indicates that local OT action in the heart is important for the protection against ischemic stress. It has also been demonstrated that in response to OT, the isolated perfused heart devoid of central nervous system influence can induce the release of atrial natriuretic peptide (ANP) (Favaretto et al., 1997; Gutkowska and Jankowski, 2012) of and nitric oxide (Danalache et al., 2007; Mukaddam-Daher et al., 2001; Wang et al., 2003). Being a highly metabolic organ, the heart is particularly vulnerable to the deleterious effects of ischemia/reperfusion (I–R). In this context, it is important that OT acts directly on cardiomyocytes (CMs) by increasing glucose uptake under normal conditions and in the presence of 2, 4-dinitrophenol, an inhibitor of energy (ATP) production (Florian et al., 2010). OT has repeatedly been demonstrated to enhance myocardial recovery following experimental I–R *in vivo* (Alizadeh et al., 2010, 2012; Das and Sarkar, 2012; Jankowski et al., 2010; Kobayashi et al., 2009) as well as in the isolated, perfused heart (Ondrejcakova et al., 2009). OT has anti-inflammatory, anti-fibrotic effects that could explain the observed myocardial protection *in vivo* (Jankowski et al., 2010).

The protection of cardiac cells through conditioning (pharmacological as well as ischemic) involves receptor-mediated signalization events that result in the salvage of mitochondrial function and subsequent cell survival (Heusch et al., 2010). Mitochondria are effectors of both I–R injury and cardioprotection exerted by OT (Alizadeh et al., 2012). We thus hypothesized that OT treatment directly protects cardiomyocyte viability through intracellular signaling

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that targets the mitochondria and prevents cell death. In this study, the experiments were designed to determine whether OT could directly protect isolated cardiomyocytes from damage induced during the acute phase of simulated I–R *in vitro* and to identify the key intracellular processes involved.

2. Materials and methods

2.1. Cell culture

Rat ventricular myoblasts (H9c2 cells) were obtained from the American Type Culture Collection (ATCC#CRL-1446, Rockville, MD, USA) and grown to 70–90% confluence in culture flasks containing Dulbecco's modified Eagle medium (ATCC 30–2002) supplemented with 10% (v/v) fetal bovine serum (Gibco Lot. 755216) and 1% (v/v) penicillin/streptomycin (Gibco 15140) at 37 °C and 5% CO_2 (Sanyo MCO-17AC incubator). Cells were detached by treatment with 0.25% trypsin with 0.1% EDTA (Multicell 325-043-EL) and cultured in 96-well plates (Corning Costar #3595) for the viability assay or 6-well plates (Corning Costar #3335 with poly-L-lysine (0.01% solution; Sigma #P4832)-treated glass cover slips (Warner Instruments CS-18R15) for immunofluorescent labeling of intracellular structures.

2.2. Antibodies

The following primary antibodies were used in this study: rabbit anti-OTR (Sigma #O4359), mouse anti-p-Akt (Ser 473, Cell Signaling #4051), rabbit anti-p-Akt (Thr308, Cell Signalling #2965S), mouse anti Cox IV (Abcam #14744), mouse anti-phospho-eNOS (Ser 1177; BD 612392), mouse anti total eNOS (BD 610296), mouse anti-Bax (Santa Cruz 7480), rabbit anti-Bcl-2 (Santa Cruz 492), p-ERK 1/2 Antibody (Thr 202/Tyr 204, Santa Cruz 16982), ERK 1/2 antibody (Santa Cruz 292838), rabbit polyclonal antibody to caveolin 3 (Abcam 2912), normal mouse IgG (Santa Cruz 2025), normal rabbit IgG (Santa Cruz 2027). Mouse anti-GAPDH (Sigma G8795) and rabbit anti-β-actin (Abcam 8227) were used as normalizing controls. Corresponding Alexa Fluor conjugated secondary antibodies were purchased from Invitrogen.

2.3. Agonists and inhibitors

Oxytocin was purchased from Sigma (O6379); OT antagonist (d(CH2)51 Tyr(Me)2 Thr4 Orn8 Tyr-NH29)-Vasotocin (OTA) from Bachem (#H-9405). Conivaptan (YM087) was a kind gift from Astellas Pharma; Wortmannin was purchased from Sigma (W1628), PKG-specific inhibitor, KT-5823, was purchased from Sigma (K1388), soluble guanylate cyclase (sGC) inhibitor, ODQ, was purchased from Sigma (O3636); particulate guanylate cyclase (pGC) inhibitor/ANP antagonist A71915 came from Bachem (H3048).

2.4. siRNA-mediated knockdown of OTR

Two different siRNA sequences were used to knockdown the expression of OTR: Qiagen # SI03106894 and Qiagen #SI03054527. H9c2 cells were plated in 24-well plates (Corning Costar # 3337) and shortly after plating, transfection complexes containing OTR or scrambled siRNA and transfection reagent (HiPerfect Qiagen # 301705, 0.25% v/v) were added to a final concentration of 5 nM siRNA. Positive (Qiagen All Stars Cell Death #SI04939025) and negative (Qiagen All Stars Negative Control AF 555 #1027286) control sequences were used in each experiment to monitor transfection efficiency.

2.5. Simulated ischemia–reperfusion

Plates with sub-confluent H9c2 cell cultures were placed in warm ischemic buffer in a sealed chamber (Billups-Rothenberg Modular Incubator Chamber, California, USA) saturated with 95% N_2 + 5% CO_2 for 2 hours at 37 °C. The ischemic buffer contained 128 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 mM HEPES, 4 mM $NaHCO_3$, pH 6.8. The volume of ischemic buffer used was the minimum volume required to coat the cellular monolayer for the prevention of cellular dehydration during the ischemic period. After 2 hours of ischemia, the plates were quickly processed and reperfusion for all conditions was performed within 3 minutes of opening the hypoxic chamber. Simulated ischemia was followed by a simulated 'reperfusion' period during which the cells were exposed to normoxic incubator-equilibrated (5% CO_2) culture medium containing 1% FBS at 37 °C for 1–4 h in the absence or in the presence of the inhibitors. Inhibitors and antagonists were provided at the induction of ischemia, dissolved in ischemic buffer, and re-administered in the reperfusion media. All controls were treated with the appropriate concentrations of vehicle.

2.6. Cell viability and death assays

Cell viability was measured by the mitochondrial reduction of the tetrazolium salt MTS [3-(3,5-dimethylthialzol-2-yl)-5-(3 carboxymethoxypheny)-2-(4-sulphophenyl)-2H-tetrazolium] into a formazan product that is soluble in culture medium (Cat. No. G3580 Promega, Montreal, QC, Canada) according to manufacturer's instructions. The colorimetric detection of formazan product was done directly in the cell culture medium using a plate spectrophotometer (Synergy 2 Multi-mode Microplate Reader, BioTek®, VT, USA). Blank absorbance values resulting from the cell-free oxidation of MTS were subtracted from all experimental values.

The Tunel (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) assay was performed using DeadEnd™ Fluorometric Tunel System (Cat. No. G3250, Promega, Montreal, QC, Canada) according to the manufacturer's protocol. Briefly, cells were grown on poly-L-lysine (0.01% solution, Sigma #P4832)-treated cover slips in 6 well plates and after the proper treatment, cells were fixed with a solution containing 3% formaldehyde and 1% methanol in PBS, pH 7.4, for 10 min at RT and permeabilized with 0.2% Triton X-100 in PBS, pH 7.4. Cells were incubated in the Tunel reaction mixture for 1 hr in the dark at 37 °C, washed and mounted with Prolong Gold anti-fade reagent with DAPI (Cat. No. P36931, Invitrogen, Montreal, QC, Canada).

Cells were visualized using an inverted light fluorescent microscope (Olympus IX51) and images were captured at 10 and 20× magnifications for analysis using a digital charge-coupled device camera (Q IMAGING #Q23774). The quantification of Tunel-positive cells as a proportion of total DAPI-stained nuclei was performed using particle analysis in ImageJ software (ImageJ (version 1.38 ×), National Institute of Health, Bethesda, MD, USA).

2.7. Intracellular ROS production

Cells were incubated with the CM-H_2DCFDA probe (**C6827**; Molecular Probes; **Invitrogen**) in warm KRH buffer (125 mM NaCl5, mM KCl, 1.8 mM CaCl2, 2.6 mM MgSO4, 5 mM HEPES, pH 7.2) at 37 °C for 30 minutes, washed and returned to normal growth conditions in the presence and absence of OT. Fluorescence was monitored at the indicated times using a fluorescence plate reader set at excitation of 485/20 nm and emission of 528/20 nm.

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