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Anti-hypertrophic effects of oxytocin in rat ventricular myocytes

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ABSTRACT

Background: Oxytocin (OT) and functional OT receptor (OTR) are expressed in the heart and are involved in blood pressure regulation and cardioprotection. Cardiac OTR signaling is associated with atrial natriuretic peptide (ANP) and nitric oxide (NO) release. During the synthesis of OT, its precursor, termed OT-Gly-Lys-Arg (OT-GKR), is accumulated in the developing rat heart. Consequently, we hypothesized that an OT-related mechanism of ANP controls cardiomyocyte (CM) hypertrophy.
Methods: The experiments were carried out in newborn and adult rat CM cultures. The enhanced protein synthesis and increased CM volume were mediated by a 24-h treatment with endothelin-1 or angiotensin II.
Results: The treatment of CM with OT or its abundant cardiac precursor, OT-GKR, revealed ANP accumulation in the cell peri-nuclear region and increased intracellular cGMP. Consequently, the CM hypertrophy was abolished by the treatment of 10 nM OT or 10 nM OT-GKR. The ANP receptor antagonist (anantin) and NO synthases inhibitor (L-NAME) inhibited cGMP production in CMs exposed to OT. STO-609 and compound C inhibition of anti-hypertrophic OT effects in CMs indicated the contribution of calcium–calmodulin kinase kinase and AMP-activated protein kinase pathways. Moreover, in ET-1 stimulated cells, OT treatment normalized the reduced Akt phosphorylation, prevented abundant accumulation of ANP and blocked ET-1-mediated translocation of nuclear factor of activated T-cells (NFAT) into the cell nuclei.
Conclusion: cGMP/protein kinase G mediates OT-induced anti-hypertrophic response with the contribution of ANP and NO. OT treatment represents a novel approach in attenuation of cardiac hypertrophy during development and cardiac pathology.

1. Introduction

The neurohypophysial hormone, oxytocin (OT), is a nonapeptide synthesized both centrally and peripherally. Within the central nervous system, the OT gene is expressed in neurons of the hypothalamic paraventricular and supraoptic nuclei [1]. OT is also synthesized peripherally in several organs including the heart [2]. To date, a single OT receptor (OTR) has been cloned from several organs. The functional OTR is expressed in the heart and is involved in blood pressure regulation [3] as well as cardioprotection associated with the activation of atrial natriuretic peptide (ANP) and nitric oxide (NO) [4,5]. In myometrial cells, the OTR is primarily associated with $G_{q/11}$ and its activation results in a phospholipase C-mediated increase in intracellular calcium, inositol trisphosphate production [6] as well as Ca^{2+}-effector proteins *like* transcription factors such as nuclear factor of activated T cells (NFAT) [7]. In the heart, recent studies recognized importance of Ca^{2+}-effector proteins, including calcineurin, calcium/calmodulin-dependent protein kinases CaMK, and mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) in hypertrophic pathways, in which NFAT plays a major role [8,9].

During the synthesis of OT, its precursor, termed OT-Gly-Lys-Arg (OT-GKR), is accumulated in the developing heart [10]. Ex vivo experiments demonstrated that the biological activity of OT-GKR included stimulation of cardiomyogenesis from stem cells [10,11] and enhancement of glucose uptake in newborn rat cardiomyocytes [12]. Recent evidence indicates that OT stimulates proliferation of some cancer cells and endothelial cells [13] in addition to inducing differentiation of embryonic stem cells to cardiomyocytes [14,15]. In mesenchymal stem cells, OT activates the Akt/ERK1/2 pathways which are involved in cell proliferation and increased the expression of genes with angiogenic and anti-apoptotic functions. OT is also beneficial in the prevention of cardiac remodeling [16]. OT, like insulin, stimulates the incorporation of amino acids into the protein of adipocytes, an effect dependent on glucose uptake [17]. Evidence further supporting the role of OT as a trophic factor is the observation that OT stimulates protein synthesis in myometrial cells by a mechanism associated with dephosphorylation of elongation factor 2 [18]. Our recent study demonstrating that glucose uptake is stimulated by OT in cardiomyocytes through the PI3K–Akt pathway [12] clearly suggests that OT may be involved in the regulation of cell hypertrophy consistent with that of insulin [19].

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The aforementioned observations led us to the hypothesis that OT and its abundant cardiac precursor OT-GKR stimulate cell hypertrophy in cardiomyocytes irrespective of the neurohormonal factors such as endothelin-1 (ET-1) and angiotensin II (AngII). We expected that OT involve PLC-mediated increase in intracellular Ca^{2+}-effector proteins or alternative actions via PI3K/Akt pathway. Experiments performed in cardiomyocyte cultures from newborn and adult rats demonstrated that in contrast to this hypothesis, OT treatment did not change protein synthesis in the cell but induced an anti-hypertrophic response in cardiomyocytes exposed to ET-1 and AngII.

2. Methods

2.1. Animals

Adult Sprague–Dawley (male, 225–250 g, 7–8 weeks old) and newborn (1 to 2-day old) Sprague–Dawley rat pups were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and with the approval of the Animal Care Committee of the Research Center of Centre Hospitalier de l'Université de Montréal (CRCHUM).

2.2. Materials

Reagents used in the study are listed in the Online supplement section.

2.3. Isolation and culture of rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVMs) were prepared from neonatal rat hearts with a kit from Worthington (Lakewood, NJ, USA) as previously reported [12].

For the isolation of adult rat ventricular myocytes (ARVMs), animals were injected intraperitoneally with 500 U heparin sulfate 15 min prior to anesthesia with sodium pentobarbital (60 mg/kg, intraperitoneally). The heart was excised, and calcium-tolerant cardiomyocytes were isolated by the Langendorff method (cardiac retrograde aortic perfusion) as previously described [20]. Briefly, all hearts were rinsed (5 ml/min) for 5 min in Krebs–Henseleit buffer (KHB) at 37 °C containing (mmol/l): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$–$7H_2O$, 1.2 KH_2PO_4, 11 dextrose, pH 7.4, and supplemented with 1.25 $CaCl_2$. The perfusion was switched to calcium-free KHB for 5 min to stop spontaneous cardiac contractions. This was followed by 20 min of perfusion with KHB supplemented with 0.05% collagenase (CLS2, Worthington Biochemical Corp), 0.03% hyaluronidase, and 0.1% BSA (Roche, fatty acid-free). For the last 5 min of perfusion, KHB was supplemented with 0.05 mM $CaCl_2$. The ventricles were then separated from the atria, minced and incubated in KHB containing collagenase (0.05%), trypsin (0.2 mg/ml), DNase I (0.2 mg/ml), $CaCl_2$ (0.1 mM), and BSA (0.1%) for 20 min at 37 °C with agitation (120 cycles per minute). The cell suspension was filtered through a 200 μm nylon mesh and centrifuged at 1000 *g* for 45 s. Cells were diluted and allowed to sediment in wash buffer solution (medium M199/KHB, 1:1). Cells were then layered on 10 ml of 6% BSA solution to separate cardiomyocytes (heavy cells) from non-cardiomyocytes (light cells). Freshly isolated cells were washed twice and diluted in plating medium M199 containing (mmol/l): creatine 5, L-carnitine 2, taurine 5, supplemented with 0.2% BSA, 1% penicillin–streptomycin and 10% fetal bovine serum (FBS). Cells were counted and plated onto ECM-coated dishes in the same plating medium at 37 °C in a humidified incubator (5% CO_2/95% air). After 2 h, the medium was changed to remove globular-shaped cells (damaged cells) and debris. The remaining calcium-tolerant rod-shaped cardiomyocytes were incubated at 37 °C for 24 h in media supplemented with or without different stimuli.

2.4. Hypertrophy measurement by [^{35}S]-methionine incorporation

The method of Devost et al. [21] was used with some modifications (see Online supplement).

2.5. Antibody details

The following primary antibodies were used in this study: Rabbit polyclonal anti-ANP prepared in the laboratory and used for radioimmunoassay [22] and immunofluorescence (lot: 17/12); Rabbit polyclonal anti-NFATC4 (nuclear factor of activated T cells) from Abcam, Cambridge, MA, USA #ab62613; Rabbit anti-Phospho-Akt (Ser473) antibody from Cell signaling (New England Biolabs, Ltd., Whitby, Ontario, Canada, #9271); Rabbit polyclonal anti-Akt from Cell signaling, #9272; Mouse IgG1, monoclonal anti-Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10) from Cell signaling #9106; and Rabbit polyclonal anti-p44/42 MAPK (ERK1/2) antibody: Cell signaling #9102.

Secondary antibody used in Western blot included: Sheep ECL anti-Mouse IgG, HRP-Linked Whole Ab from GE Healthcare Life Sciences Baie d'Urfe, Quebec, Canada, #NA931V and Donkey ECL anti-Rabbit IgG, HRP-Linked Whole Ab, GE Healthcare Life Sciences #NA934V.

For detection of primary antibody complexes by immunofluorescence, the following conjugates were used: Alexa Fluor 594 donkey anti-rabbit IgG #A21207 from Invitrogen, Burlington, Canada; Alexa Fluor 594 donkey anti-mouse IgG #A21203, Invitrogen; Alexa Fluor 488 donkey anti-rabbit IgG #A21206, Invitrogen; and Alexa Fluor 488 donkey anti-mouse IgG #A21202, Invitrogen.

2.6. ANP measurements by radioimmunoassay

Cardiomyocytes were grown in 24-well plates in DMEM low glucose medium supplemented with 10% FBS. Beating cells were washed once with serum-free medium and starved overnight with 0.2% FBS before any treatment. On the day of the experiment, the medium was changed to 1% FBS and different stimulations were processed for 24 h. An aliquot of medium was taken, and protease inhibitors were added and stored at −80 °C for radioimmunoassay as previously reported [22].

2.7. cGMP measurement

After stimulation with ET-1 for 30 min in the presence or absence of 15 min pretreatment with OT or OT-GKR, the assay was performed by ELISA kit according to the manufacturer's instructions (Cell Signaling Technology, Inc. MA, USA) or by radioimmunoassay according to a previously reported method [23,24].

2.8. Immunofluorescence staining

The details of the experiments were already reported [12,16]. For detection, appropriate secondary antibodies conjugated with Alexa Fluor were used, and the nuclei were counterstained with DAPI Prolong Gold antifade reagent from Invitrogen (Molecular Probes) #P36935.

2.9. Western blot analysis

The materials and methodology of phospho-Akt and phospho-ERK1/2 were performed according to our recent paper [16]. Briefly, at the end of different stimulations, the cells were washed twice with cold PBS buffer and lysed in a buffer containing 25 mM Tris–HCl, pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS). The lysate was centrifuged for 20 min at 12,000 *g* at 4 °C to remove insoluble material, and the resulting supernatant was taken for immunoblotting with 10 μl saved for protein measurements with a BCA kit (Pierce Biotechnology, IL, USA).

Equal amounts of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. The membranes were first blocked for 1 h with 5% (w/v) milk in TBS-Tween (TBST), pH 7.4, containing 150 mM NaCl, 20 mM Tris, and 0.05% Tween 20. They were then incubated overnight at 4 °C with the primary antibodies for detection of phosphorylated proteins, followed by 3 × 5 min washing out of free antibodies with TBST and incubated for 1 h at room temperature (RT) with the appropriate secondary antibodies conjugated to horseradish peroxidase. Antigen–antibody complexes were detected by the enhanced chemiluminescence method (ECL). The membranes were washed again and incubated for 1 h at RT for detection of total (non-phosphorylated) proteins. The membranes were incubated as before with second antibodies and developed with ECL method. Quantitative analysis of the scanned films was performed with the public ImageJ program (NIH, USA).

3. Statistical analysis

The data are expressed as mean ± SEM. Variables that were not normally distributed were analyzed after appropriate transformations. For calculations we have used GraphPad Prism 5 Software (San Diego, CA). The analysis in the groups was performed by one-way ANOVA and nonparametric analyses for repeated measures with Bonferroni post hoc test. In the figures, the $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ significance of differences were expressed as single, double and triple symbols, respectively.

4. Results

4.1. Methionine incorporation and ANP release in NRVMs stimulated with OT

To examine whether the OT treatment of rat neonatal cardiomyocytes stimulates hypertrophy we measured [^{35}S]-methionine incorporation by cells exposed to OT (10–1000 nM) or its elongated form, OT-GKR for 24 h. As demonstrated in Fig. 1A and B, treatment with OT or OT-GKR did not increase [^{35}S]-methionine incorporation. In contrast, [^{35}S]-methionine was incorporated into cells exposed to 10 nM of insulin and this result was not changed when insulin was combined with 10 nM of OT or OT-GKR (Fig. 1C). ANP which is released from cardiomyocytes in response