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AUTOCRINE OXYTOCIN ACTION IN CARDIOMYOCYTES: DUAL STIMULATION OF ANP SECRETION AND NITRIC OXIDE-DEPENDENT INHIBITION

AÇÃO AUTÓCRINA DA OCITOCINA EM CARDIOMIÓCITOS: DUPLA ESTIMULAÇÃO DA SECREÇÃO DE ANP E INIBIÇÃO DEPENDENTE DE ÓXIDO NÍTRICO

ACCIÓN AUTOCRINA DE LA OXITOCINA EN CARDIOMIOCITOS: ESTIMULACIÓN DUAL DE LA SECRECIÓN DE ANP E INHIBICIÓN DEPENDIENTE DEL ÓXIDO NÍTRICO

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ABSTRACT

Historically known for its effects on the reproductive system, oxytocin (OT) is now known to contribute to the regulation of cardiovascular and electrolyte homeostasis. OT is produced in the supraoptic and paraventricular nuclei of the hypothalamus and released into the plasma from neural terminals in the posterior pituitary. However, many studies have identified extracerebral sites of OT production, including the heart and vascular endothelium. Activation of its receptors on endothelial cells, as well as in the hypothalamic/pituitary and cardiac systems, can result in the production of nitric oxide (NO). The present study aimed to investigate the role of NO in the regulation of OT-stimulated atrial natriuretic peptide (ANP) secretion in primary cultures of mouse embryo cardiomyocytes. For this purpose, hearts from Balb C mouse embryos, 19 to 21 days of intrauterine life, were isolated and cultured for assays with OT and other substances that interfere with the synthesis of NO and its second messenger cGMP. The addition of increasing concentrations of OT (0.1, 1, 10, and 100 μM) induced a proportional increase in the secretion of ANP and nitrate into the medium, confirming the stimulatory action of OT in cardiomyocytes. The blockade of ANP release stimulated by OT (10 μM) was observed after the addition of Ornithine Vasotocin (CVI-OVT) (100 μM), a specific OT antagonist. This antagonist inhibited basal ANP secretion when added individually, suggesting that OT may act via an autocrine, tonic stimulatory mechanism on ANP secretion. Amplification of OT-stimulated ANP secretion (10 μM) was observed after

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its combination with L-NAME, a nitric oxide synthase (NOS) inhibitor (600 μM), and ODQ (100 μM), a soluble guanylate cyclase inhibitor, suggesting the occurrence of nitrergic negative feedback on OT-stimulated ANP release in cardiomyocytes. The results obtained demonstrated nitrergic inhibitory modulation of OT-stimulated ANP secretion.

Keywords: Oxytocin. Atrial Natriuretic Peptide. Nitric Oxide. Cardiomyocytes.

RESUMO

Historicamente conhecida por suas ações sobre o sistema reprodutor, hoje se sabe que a ocitocina (OT) também pode contribuir para a regulação da homeostase cardiovascular e hidroeletrolítica. A OT é produzida nos núcleos supra-óptico e paraventricular do hipotálamo e liberada para o plasma a partir de terminais neurais da pituitária posterior, no entanto, muitos estudos identificaram locais extra-cerebrais de produção OT, incluindo o coração e o endotélio vascular. A ativação de seus receptores em células endoteliais, bem como em sistemas hipotalâmicos/hipofisários e cardíaco, pode resultar na produção de óxido nítrico (NO). O presente trabalho teve como objetivo verificar o papel do NO na regulação da secreção de peptídeo natriurético atrial (ANP) estimulada por OT em cultura primária de cardiomiócitos de embriões de camundongos. Para tal, corações de embriões de camundongos Balb C, com 19 a 21 dias de vida intra-uterina, foram isolados e cultivados para os ensaios com OT e demais substâncias interferentes na síntese de NO e GMPc seu segundo mensageiro. A adição de concentrações crescentes de OT (0.1, 1, 10 e 100 μM) induziu aumento proporcional na secreção de ANP e nitrato para o meio, confirmando a ação estimuladora da OT em cardiomiócitos. O bloqueio da liberação de ANP estimulada por OT (10 μM) foi observada após adição de Ornitina Vasotocina (CVI-OVT) (100 μM), um antagonista específico de OT. Este antagonista inibiu a secreção basal de ANP, quando adicionado individualmente, sugerindo que a OT pode atuar via mecanismo autócrino, tônico estimulatório sobre a secreção de ANP. Amplificação da secreção de ANP estimulada por OT (10 μM) foi observada após sua associação com L-NAME, um inibidor da sintase de óxido nítrico (NOS) (600 μM), e ODQ (100 μM), um inibidor da guanilato ciclase solúvel, sugerindo a ocorrência de feedback negativo nitrérgico na liberação de ANP estimulada por OT no cardiomiócito. Os resultados obtidos mostraram modulação nitrérgica inibidora sobre a secreção de ANP estimulada por OT.

Palavras-chave: Ocitocina. Peptídeo Natriurético Atrial. Óxido Nítrico. Cardiomiócitos.

RESUMEN

Históricamente conocida por sus efectos sobre el sistema reproductivo, ahora se sabe que la oxitocina (OT) contribuye a la regulación de la homeostasis cardiovascular y electrolítica. La OT se produce en los núcleos supraóptico y paraventricular del hipotálamo y se libera al plasma desde las terminales neuronales en la hipófisis posterior. Sin embargo, muchos estudios han identificado sitios extracerebrales de producción de OT, incluyendo el corazón y el endotelio vascular. La activación de sus receptores en las células endoteliales, así como en los sistemas hipotálamo/hipofisario y cardíaco, puede resultar en la producción de óxido nítrico (NO). El presente estudio tuvo como objetivo investigar el papel del NO en la regulación de la secreción del péptido natriurético auricular (ANP) estimulada por OT en cultivos primarios de cardiomiocitos de embriones de ratón. Para este propósito, corazones de embriones de ratón Balb C, de 19 a 21 días de vida intrauterina, fueron aislados y cultivados para ensayos con OT y otras sustancias que interfieren con la síntesis de NO y su segundo mensajero cGMP. La adición de concentraciones crecientes de OT (0,1, 1, 10 y

100 µM) indujo un aumento proporcional en la secreción de ANP y nitrato al medio, confirmando la acción estimuladora de OT en cardiomiocitos. El bloqueo de la liberación de ANP estimulada por OT (10 µM) fue observado después de la adición de Ornitina Vasotocina (CVI-OVT) (100 µM), un antagonista específico de OT. Este antagonista inhibió la secreción basal de ANP cuando fue añadido individualmente, sugiriendo que OT puede actuar a través de un mecanismo estimulador tónico autocrino sobre la secreción de ANP. Se observó una amplificación de la secreción de ANP estimulada por OT (10 µM) tras su combinación con L-NAME, un inhibidor de la óxido nítrico sintasa (NOS) (600 µM), y ODQ (100 µM), un inhibidor soluble de la guanilato ciclasa, lo que sugiere la presencia de retroalimentación negativa nitrérgica en la liberación de ANP estimulada por OT en cardiomiocitos. Los resultados obtenidos demostraron una modulación inhibitoria nitrérgica de la secreción de ANP estimulada por OT.

Palabras clave: Oxitocina. Péptido Natriurético Auricular. Óxido Nítrico. Cardiomiocitos.

1 INTRODUCTION

Classically known for its actions on the reproductive system, it is now known that OT can also contribute to the regulation of cardiovascular and hydroelectrolytic homeostasis (GUTKOWSKA; JANKOWSKI, 2012).

OT is a nonapeptide hormone, with a molecular weight of 1007 KDa. It is synthesized as a prohormone (pro-ocytophysin) by the magnocellular neurons of the paraventricular (NPV) and supraoptic (NSO) nuclei of the hypothalamus and released into the circulation from the neurohypophysis. It has an average life in the systemic circulation of approximately 5-10 minutes. The cyclic part of its molecule, with a disulfide bond (-S-S-), is fundamental to exert its biological effects (ANTUNES-RODRIGUES et al., 2004).

The most potent stimuli for OT release are: increased plasma osmolality, blood pressure and hypervolemia (KADEKARO et al., 1992). In the heart, OT stimulates the release of ANP and decreases the force and frequency of contraction, leading to an effective decrease in cardiac output and, consequently, in blood pressure. This action may be dependent, in part, on the synthesis of NO (MUKADDAM-DAHER et al., 2001) or on the release of ANP (FAVARETTO et al., 1997), since both substances stimulate the synthesis of cGMP, although by different enzymes guanylate cyclase (CG) (FAVARETTO et al., 1997). cGMP can inhibit cardiac contractility by decreasing calcium currents generated via L-type channels (TOHSE et al., 1995). These currents participate in the release of ANP under various stimulated conditions (REBSAMEN et al., 1997; JIN et al., 2004), suggesting that cGMP can inhibit the release of ANP by reducing these currents. In addition, the use of N(G)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of the enzyme nitric oxide synthase (NOS) (FRANDSEN et al., 2001; SANDER et al., 1999), resulted in an additive effect on angiotensin II (ANG II)-induced ANP release, which was attenuated by NO or 8-bromine cGMP treatments in rat perfused atria (SOUALMIA et al., 2001), suggesting that NO may act by inhibiting ANP release induced by ANGII.

The mechanisms of action of NO in the heart cell are mediated by the increase in the concentration of cGMP. NO targets the enzyme CGs in the heart cell. Once activated, CGs catalyzes the conversion of GTP into cGMP, which consequently activates protein kinase G (PKG). PKG can inhibit the influx of calcium into the cell, which occurs through voltage-gated calcium channels present in the cell membrane (HARTZELL; FISCHMEISTER, 1986; MERY et al., 1991) and may decrease the sensitivity of myofilaments to calcium (SHAH et al., 1994; YASUDA; LEW, 1997), probably through the phosphorylation of Troponin I (TnI) (LAYLAND

et al., 2005), resulting in a decrease in contractile force and attenuating adrenergic effects (LAYLAND et al., 2002).

In addition, there is evidence that NO blocks the action of phospholipase C, consequently inhibiting the release of calcium mediated by inositol triphosphate (IP3) (HIRATA et al., 1990; CLEMENTI et al., 1995). PKG also inhibits the release of calcium from the sarcoplasmic reticulum (SR) by phosphorylating and inhibiting the IP3 receptors present in its membranes (KOMALAVILAS; LINCOLN, 1996). In addition, NO induces increased calcium transport by the sarcoplasmic reticulum calcium pump (SERCA) independently of cGMP (BUSSE; FLEMING, 2000) and also seems to increase phospholamban protein phosphorylation during vascular relaxation (KARCZEWSKI et al., 1998). All these effects determine the decrease in the concentration of free calcium in the cytoplasm and consequently contribute to muscle relaxation.

These effects attributed to NO have an autocrine character in the cardiomyocytes themselves, through various stimuli (HAN et al., 1998; ZIOLO et al., 2001; BAROUCH et al., 2002), but it may also be a paracrine effect of NO, produced by NOS3 in endothelial cells (BALLIGAND; CANNON, 1997). On the other hand, the NO produced by NOS expressed in neuronal varicosities determines the decrease in the release of norepinephrine and potentiates the release of acetylcholine, reinforcing the anti-adrenergic modulation in the heart (PATON et al., 2002). Endogenous NO has other metabolic and ionic effects through cGMP-independent pathways, such as inhibition of mitochondrial respiration, inhibition of glucose transport, increased transport of free fatty acids, and activation of the Na+ K+ ATPase pump in cardiomyocytes (MASSION et al., 2003). The mechanism by which NO is removed from GCs after the required vasodilation has occurred is unknown. It is known that cGMP production is interrupted seconds after the removal of NO from the soluble guanylate cyclase enzyme (BECKMAN; KOPPENOL., 1996).

Oxytocin exerts a stimulating effect, proportional to the dose applied, on ANP secretion directly in the heart under in vitro study, and this effect is reversed by the action of a specific OT antagonist. Similar to ANP, OT also has a negative inotropic and chronotropic effect, effects that are also mimicked by cGMP, the main mediator of ANP actions, probable intermediary of OT actions in the heart (FAVARETTO et al., 1997; GUTKOWSKA et al., 1997).

However, the physiological role of cardiovascular OT production is poorly known, some evidence supports the hypothesis that OT may act by decreasing cardiac activity by negative chronotropic and inotropic effect. This action may be dependent, in part, on the synthesis of

nitric oxide (NO) (MUKADDAM-DAHER et al., 2001) or on the release of ANP (FAVARETTO et al., 1997). In addition, evidence shows that OT, NO and ANP act on the cardiovascular system by increasing the production of cGMP. This in turn is used as a mediator of vascular relaxation, negative inotropic and chronotropic effects on the heart (JANKOWSKI et al., 1998).

These evidences lead us to the hypothesis that OT may participate in intrinsic mechanisms of cardiac function regulation as an endocrine, autocrine and/or paracrine signal, mediating inhibitory effects of contractile activity and stimulators of ANP secretory activity and NO synthesis, however, a direct action still needs to be confirmed. Additionally, the fact that NO acts as a possible inhibitory modulator of ANP release (LESKINEN et al., 1995) and that its enzymes are present in the heart (BALLIGAND et al., 1995) prioritizes the study of mechanisms exclusive to cardiomyocytes involving OT and possible stimuli mediated by it on ANP secretion and NO synthesis from nitrate, a metabolite resulting from its degradation in primary culture of cardiomyocytes. This study aims to study possible nitrergic mechanisms related to the regulation of ANP secretion by oxytocin in primary culture of cardiomyocytes from mouse embryos.

2 METHODOLOGY

2.1 ANIMALS

In this study, pregnant female Balb C mice (19 to 21 days) from the Vivarium of the Neuroendocrinology Laboratory of the Medical School of Ribeirão Preto (FMRP/USP) were used. With an approximate weight of 50 □ 10 g, kept in community cages, in an artificially controlled environment (24±2 ºC; 14 hours of light and 10 hours of darkness) with availability of water and feed ad libitum.

2.2 CARDIOMYOCYTE CULTURE

All experiments were conducted based on a primary cardiomyocyte culture model (MACHADO et al., 2000). It is a simple culture of cardiac cells, predominantly from mouse embryo myocytes (± 21 days). Cells are demanding in terms of nutritional metabolic demand, but resist well for several days without changing media (± 8 days).

The hearts of embryos of Balb C mice with 19 to 21 days of intrauterine life were removed under anesthesia with ethyl ether for culture in each experiment. Then fragmented in refrigerated Ringer's solution, washed 3 times in a row in 0.1 % phosphate buffer solution

(PBS), without Ca2+ and Mg2+, and dissociated in 0.01% collagenase solution (type II) (Worthington Biochemical Corp) + 0.01 % trypsin (Gibco-Brl) in PBS (0.1 %) by magnetic stirring (10 repetitions of 5 min) at 37ºC, pH=7.4. A total of 9 cell suspensions (10 ml) were collected after the first cell suspension was discarded between the changes. These suspended cells were added to 0.5 ml of refrigerated fetal bovine serum (Gibco) (inactivated at 56 ºC/15 min), then centrifuged for 10 min (4 ºC at 1,200 rpm) and the precipitate recovered in a single flask with 18 ml of DMEM (Dulbecon's modified Eagles´s medium) enriched with fetal bovine serum (FBS) (5%); inactivated horse serum (SH) (10%) (56ºC/15min) and chick embryo extract (1%). After a new centrifugation (10 min at 4ºC at 1,200 rpm), the precipitate was resuspended in 2 ml of the same medium and an aliquot of 10 µl was removed for cell counting. Approximately 1.2 x 105 cells were seeded in 24-well (Corning) culture plates containing 1 ml of enriched DMEM. For total adhesion of the cells, the surface of the well was covered with a 0.001 % pig gelatin film. A media change was made 24 hours later when the viability of the crop was evaluated. In this condition, most of the cells have already shown discrete heartbeats, which tended to increase in intensity with the advance of the days and their growth in a greenhouse programmed for an atmosphere of 5% CO2 (mixed with atmospheric air) at 37 ºC.

2.3 EXPERIMENTAL DESIGN

Cardiomyocytes were incubated for 96 hours with enriched DMEM (5% (SFB) and 10% HS). After this time, the medium was replaced with serum-free DMEM and incubated for 60 minutes to normalize baseline ANP levels. Then another 15 min for the test with test substances. This time sequence was followed for all experiments. Two culture plates were submitted, with the first column being reserved for control and the rest of the columns for test substances. The incubation medium was removed from each interval and stored in a freezer at -70 °C for quantification of ANP and nitrate.

2.4 ANP QUANTIFICATION

The quantification of ANP was performed by radioimmunoassay with double antibody according to the method described by GUTKOWSKA et al. (1984), at the Laboratory of Neuroendocrinology at the Medical School of Ribeirão Preto (FMRP/USP).

The radioimmunoassay consists of the preparation of a standard curve, made by testing 100 µl of ANF standards with serial dilution from 0.76 to 195.3 pg/tube (1-28-

Peninsula®), 100µl of ANP buffer [Na2HPO4 7 H2O – bibasic sodium monophosphate heptahydrate, 134.04 g/l (Dynamics Reagents®); NaH2PO4 . H2O - monobasic sodium phosphate monohydrate, 69 g/l (Dynamics Reagents®); NaCl - sodium chloride, 2.92g/l (Dynamic Reagents®); Bovine Albumin Fraction V, 1.0 g/l (SIGMA® A-8022); Triton X-100, 1.0ml (SIGMA® T-8787); Polyethylene glycol, 6.25% (PEG PM 6000 - REAGEN®) and NaN3 – sodium azide, 0.01% (SIGMA® S-8032); 100µl of primary antibody (anti-rat rabbit ANF, anti-rat anti-rat aggregabulin produced in rabbit anti-rat alpha ANF, dilution 1:60,000- achieved by immunization at FMRP/USP), diluted with 1% normal rabbit serum, 100µl of labeled hormone (125I- ANF (1-28) and the reaction was completed with 100µl of second antibody (anti-rabbit goat ANF, ane rabbit maglobulin produced in goats, dilution of 1:20,000 – manufactured by immunization at FMRP/USP), followed by overnight incubation of 4 days at 4 ºC. At the end, 1 ml of 6.25% polyethylene glycol (PEG PM 6000 - REAGEN®) refrigerated was added to accelerate the precipitation of the complex formed. It was centrifuged in a refrigerated centrifuge at 4 ºC (Mod. 3 K10 – SIGMA)® for 25 min at 3,000 rpm and the supernatant was aspirated and the radioactivity of the precipitate was counted in cpm on the Gamma counter (Cobra 5002-PACKARD□) with counting efficiency greater than 72%.

The ANP concentrations in the samples were obtained from the standard curve previously described. Samples from the same group were quantified in a single assay to avoid inter-assay variations. The samples were quantified in duplicate, using two standard curves, one read at the beginning and the other at the end of the reading of the test, for pipetting control. These two procedures are also important for intra-assay control.

2.5 NITRATE (NO3) DOSAGE

The determination of nitrate concentration was also performed at the Laboratory of Neuroendocrinology of the Medical School of Ribeirão Preto (FMRP/USP) by the chemiluminescence technique, through a nitric oxide analyzer (Nitric Oxide Analyzer, NOATM280, Sievers Instruments, Inc., Colorado, USA), according to the method described by HAMPL et al. (1996).

After each experimental protocol, aliquots of 5 □l of these samples were injected into the reaction chamber where the nitrate reacts with the vanadium chloride forming NO, which in turn reacts with the O3 forming nitrogen dioxide (NO2-). NO2- is in an unstable form and has the ability to emit photons that crash against a photosensitive surface of a photomultiplier

cell. This generates electron current that is captured, amplified and processed by a digital analog transducer, giving rise to the graphic tracing. The area contained in the route is then demarcated and quantified by the NOAnalysisTM Software (version 3.00PNE) program operating exclusively with the analysis system. The correlation coefficient for the standard curves ranged from 0.98 to 1.0 and the minimum detectable dose was 0.5 μM.

2.6 STATISTICAL ANALYSIS

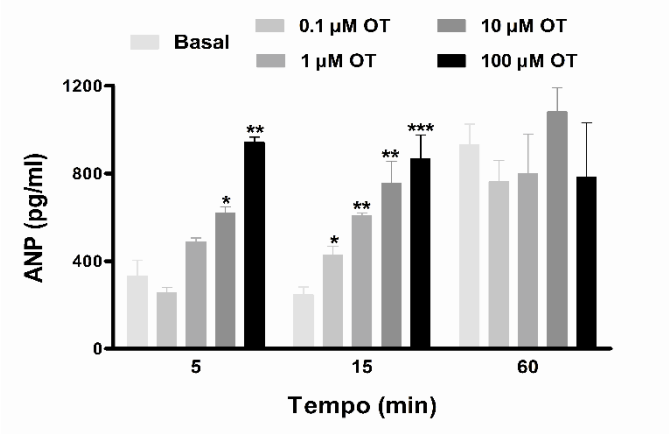
The results are presented as mean ± mean standard error (EPM). The statistical test applied was ANOVA followed by the Newman-Keuls test or Student's t-test, for comparisons between groups. The determinations of statistically significant differences took into account $p<0.05$ (* $p<0.05$, ** $p<0.01$ for comparisons with the control group and + $p<0.05$, ++ $p<0.01$ for comparisons between treated groups.

3 RESULTS

Regarding the effects on baseline concentration of ANP and nitrate, OT administration induced a dose-dependent increase (0.1, 1, 10, and 100 μM) in ANP release to the medium at 5 and 15 min (Figure 1). The effects of OT on ANP release began at 5 min after the addition of the 10 and 100 μM doses, and were maintained at 15 min, when all doses induced a significant increase, and at 60 minutes, when no more significant changes were detected. On the other hand, dose-dependent nitrate release occurred only in 15 min for all doses of OT tested (Figure 2).

Figure 1

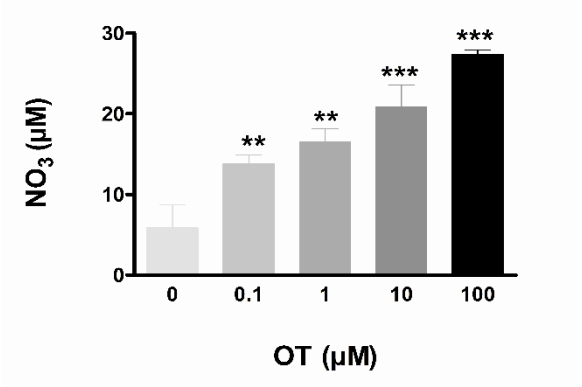
Effects of oxytocin (OT) administration on ANP release (n=6). Values are presented as average±EPM. p<0.05; ** p<0.01; p<0.001 for comparisons between treated versus baseline in the same cell group*



Source: Conde et al., 2024.

Figure 2

*Effects of oxytocin (OT) administration on nitrate (NO3) release after 15 min of treatment. Values are presented as average±EPM. ((n=6) * p<0.05; ** p<0.01; *** p<0.001 for comparisons between treated versus baseline in the same group of cells*



Source: Conde et al., 2024.

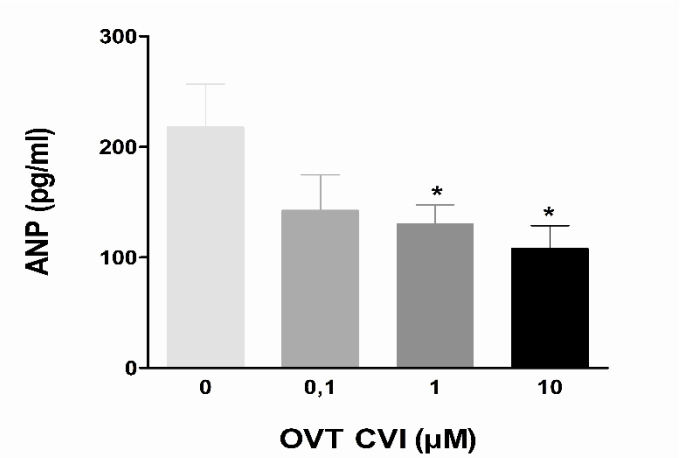
To verify the occurrence of some intrinsic effect of cardiac OT on ANP secretion, CVI-OVT (0.1, 1.10 µM), a specific antagonist for OT receptors, was added. A significant decrease in ANP release occurred only after administration of the 1 µM dose, but with no difference in magnitude with a 10-fold higher concentration, after 15 min of observation (Figure 3), suggesting a limited effect by continuous and low constitutive OT release into the medium.

Regarding the effects of OT receptor blockade on OT-stimulated ANP secretion, there was a decrease in ANP release 15 min after the addition of the antagonist individually at a

dose of 100 μM. When associated with OT, the blockade (CVI-OVT 100 μM) of oxytocinergic stimulation on ANP release was demonstrated (Figure 4). This substance was effective in inhibiting OT-induced ANP release (10 μM). Proving the specificity of OT effects.

Figure 3

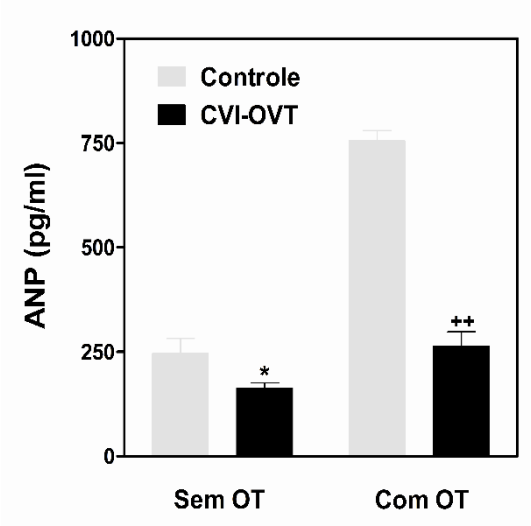
Effects of individual OT antagonist administration on baseline ANP release after 15 min of treatment. Values are presented as average±EPM. p<0.05; ** p<0.01 for treated-versus-control comparisons*



Source: Conde et al., 2024.

Figure 4

Effects of CVI-OVT administration (100 μM) on basal ANP release (without OT) or OT-induced (with OT) under equimolarity conditions (10 μM) after 15 min of treatment. Values are presented as average±EPM. p<0.05; ** p<0.01 for treated-versus-control comparisons. + p<0.05; ++ p<0.01 for comparisons between treated groups versus respective controls*



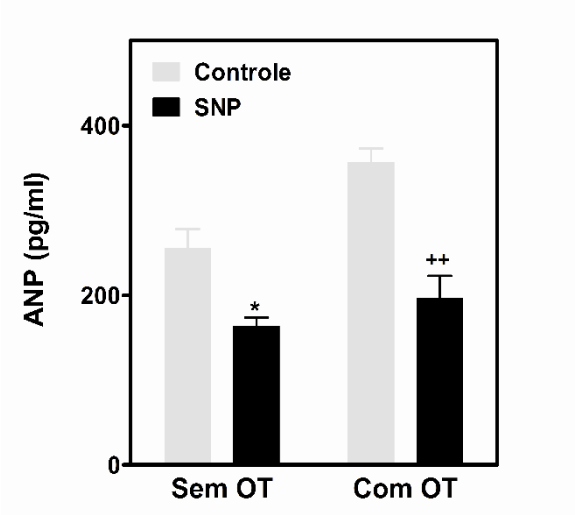
Source: Conde et al., 2024.

To verify possible effects of increased NO production on basal or OT-induced ANP secretion, SNP (600 μM), a NO donor, was added individually or in association with OT (10 μM). A significant decrease in ANP release was obtained after the addition of the NO donor, in both experimental conditions after 15 min of observation (Figure 5).

In order to study possible effects of decreased NO production on basal or OT-stimulated ANP secretion (10 μM), L-NAME (600 μM), a nonspecific NOS inhibitor, was used. Significant amplification of OT-stimulated ANP secretion was observed after 15 min of observation (Figure 6).

Figure 5

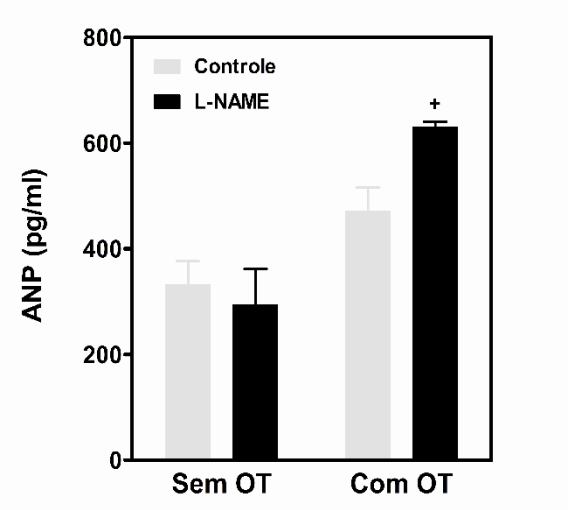
*Effects of SNP administration (600 μM) on baseline (no OT) or OT-stimulated (10 μM) ANP release after 15 min of treatment. The values are presented as average±EPM (n=6). p *<0.05; ** p<0.01 for treated-versus-control comparisons. + p<0.05; ++ p<0.01 for comparisons between treated groups versus respective controls*



Source: Conde et al., 2024.

Figure 6

Effects of administration of L-NAME (600 μM), a nonspecific NOS inhibitor, on baseline (no OT) or OT-stimulated (10 μM) ANP release (with OT) after 15 min of treatment. The values are presented as average±EPM (n=6). + p<0.05; ++ p<0.01 for comparisons between treated groups versus respective controls

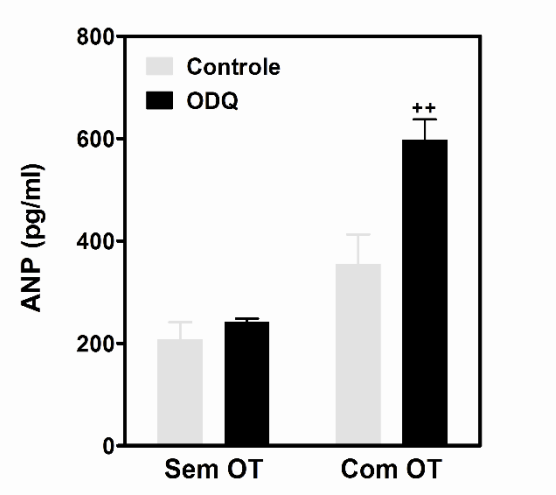


Source: Conde et al., 2024.

We sought to evaluate the participation of cGMP in the nitrergic modulation of basal or OT-stimulated ANP secretion (10 μM) by adding a GCs inhibitor (100 μM ODQ). An additive effect of this substance was observed only on OT-stimulated ANP secretion (Figure 7).

Figure 7

Effects of administration of ODQ (100 µM), a CGs inhibitor, on baseline (no OT) or OT-stimulated (10 µM) ANP release after 15 min of treatment. The values are presented as average±EPM (n=6). + p<0.05; ++ p<0.01 for comparisons between treated groups versus respective controls



Source: Conde et al., 2024.

4 DISCUSSION

The results confirmed, in isolated cardiomyocytes, the stimulatory action of OT on ANP secretion (Figure 1), as evidenced by other *in vivo studies* (GUTKOWSKA et al., 1997) and, *in vitro*, in isolated heart preparation (FAVARETTO et al., 1997). The decrease in basal ANP secretion and the nullification of the OT stimulatory effect, resulting from the addition of a specific antagonist, suggests the specificity of hormonal action and the occurrence of a tonic-stimulatory regulation of OT, in an atypical autocrine regulation (Figure 3). However, we were unsuccessful in the attempt to measure the concentration of OT in the medium (data not shown), suggesting that this action depends on particular regulatory conditions to occur, since the synthesis of OT by the cardiomyocyte has been evidenced by other studies (JANKOWSKI et al, 1998; GUTKOWSKA et al., 2000; PETERSSON et al, 2002)

The increase in OT-stimulated ANP secretion was accompanied by a proportional increase in nitrate in the medium, relating NO production to these effects (Figure 2). This antagonist also induced a significant decrease in baseline ANP release when administered alone, suggesting the occurrence of autocrine regulation of OT by a stimulatory tonic mechanism.

In fact, these results confirm OT effects on ANP release demonstrated by some studies. Plasma ANP concentrations increased after ip (20 min) or iv (5 min) injection of OT

in awake rats. These changes were related to increased urinary osmolarity, natriuresis, and calliuresis induced by OT injection via i.p. (HAANWINCKEL et al., 1995). In addition, OT also promoted an increase in the concentration of ANP in the effluent, an effect that was completely blocked by perfusion with an OT receptor antagonist, which resulted in a reduction in the concentration of ANP in the effluent to values below baseline (GUTKOWSKA et al., 1997). Reinforcing these data, studies have shown that the incubation of atria isolated from rats with OT (1μM) resulted in an increase in ANP secretion, an effect once again blocked by an oxytocinergic receptor antagonist, which also maintained ANP secretion at levels below control (FAVARETTO et al., 1997). Our results also reproduced the effects of the OT antagonist in reducing ANP release in cardiomyocytes to values below baseline (Figure 10), which suggests the participation of a cardiac oxytocinergic system in the control of ANP atrial secretion (FAVARETTO et al., 1997; GUTKOWSKA et al., 1997).

Subsequent studies have shown that OT is synthesized, stored, and released from cardiomyocytes. The OT content measured in cell cultures and collected after 1 h of incubation averaged 228 ± 55 pg/ml from the medium and 53 ± 3 pg/mg protein in the cells. These authors verified the expression of OT-specific mRNA in different mouse heart chambers (JANKOWSKI et al., 1998). In addition, the levels of mRNA for OT receptors in the atria were found to be in greater quantity than in the ventricles, overall the mRNA levels of OT receptors were calculated to be at least 10 times lower than the level of OT receptor mRNA present in the uterus of rats (JANKOWSKI, et al 1998).

However, it is possible that unknown factors modulate the synthesis and cardiac secretion of oxytocin, controlling the tissue concentration of this peptide, which could thus reach high levels, capable of influencing the electrical and mechanical function of the heart, in addition to its endocrine function, considering the fact that mice depleted of the oxytocin gene have high intrinsic heart rate (MICHELINI et al., 2003).

The use of an NO synthesis blocker (L-NAME) potentiated the secretion of ANP stimulated (but not basal) by OT (Figure 6) and, in contrast, the use of a donor (SNP) inhibited both basal and stimulated secretion (Figure 5), confirming in cardiomyocytes, the inhibitory regulation of NO over ANP release.

In fact, these results confirmed effects of NO in inhibiting ANP release as demonstrated by *in vivo studies.* They showed that NO released from the endocardium inhibited ANP secretion, since the use of a NO synthesis blocker (L-NAME) resulted in increased basal or stretch-induced ANP secretion (LESKINEN et al., 1995). Other evidence

showed that NO inhibited atrial mRNA expression for ANP (LEE et al., 2000). These results show that the simple inhibition of NO synthesis is able to stimulate the release of ANP, suggesting a tonic, NO-inhibiting mechanism over the basal or stimulated release of ANP. It is pertinent to note, however, that the effect of inhibition of NO synthesis was more effective in the OT-stimulated condition, suggesting that some intrinsic inhibitory mechanism may be limiting NO production in the constitutive condition.

The mechanism used by NO to produce this inhibitory effect may depend on the synthesis of cGMP via activation of the soluble guanylate cyclase enzyme, as demonstrated in our results by the increased release of ANP resulting from the addition of ODQ, a specific inhibitor of this enzyme.

cGMP predominantly regulates cell metabolism in an inhibitory manner by negatively interfering with signaling that increases the concentration of free calcium ion in the cytoplasm (DOMEK-LOPACINSKA; STROSZNAJDER., 2005), which results in a decrease in free calcium in the cytoplasm (TOHSE et al., 1995),

The increase in nitrate concentration (baseline) and the amplification of OT-induced ANP release after administration of L-NAME (Figure 6) or ODQ (Figure 7) observed in our results indicate that NO may act by a negative feedback via cGMP production limiting ANP release induced by low OT concentrations (baseline cardiac OT concentrations).

This hypothesis is corroborated by evidence that demonstrated a negative chronotropic and inotropic effect on an isolated heart in spontaneous activity (FAVARETTO et al., 1997). Effects mimicked by the addition of cyclic 8-bromoguanosine monophosphate (8-bcGMP), suggesting that cGMP acted mediating these responses (FAVARETTO et al., 1997).

However, it is difficult to think that the inotropic and chronotropic effects are negative and result from a direct action of OT on cardiomyocytes. After all, the OT receptor is coupled to the Gq protein and, consequently, its activation results in the stimulation of phospholipase C, generation of inositol-triphosphate (IP3) and diacylglycerol, resulting in the release of the calcium ion from intracellular reserves (GIMPL; FAHRENHOLZ, 2001). However, it is possible that the activation of oxytocinergic receptors in cardiomyocytes results in the activation of the enzyme NOS and increased synthesis of nitric oxide (NO) which, in turn, can stimulate the enzyme soluble guanylate cyclase, resulting in greater synthesis of cGMP in these cells. In fact, an increase in both NO and cGMP levels results in a negative inotropic effect, which can be explained based on the activation of cGMP-dependent protein kinases,

which inhibit ATP synthesis and close voltage-gated calcium channels (BRUTSAERT, 2003). In addition, it is possible that other pathways used by cGMP may activate phosphodiesterase II (PEDII) that would reduce the cytosolic concentration of cAMP, which would promote the inhibition of protein kinase A (PKA) with consequent negative inotropic and chronotropic effect (MASSION et al., 2003). In addition to these cardiac effects, OT and ANP acted by promoting increased excretion of renal sodium and potassium by mechanisms dependent on increased cGMP synthesis (SOARES et al., 1999).

Further investigations revealed that ANP secretion stimulated by ANG II was also amplified by L-NAME, and attenuated by treatment with NO or 8-bcGMP. In addition, increases or decreases in cGMP levels occurred after treatment with ANG II or L-NAME, respectively (SOUALMIA et al., 2001). These results show that similarity to OT, NO and cGMP can negatively modulate ANG II effects, suggesting the existence of a negative feedback on the release of ANP induced by this hormone. Furthermore, the fact that cGMP 8-bromine did not induce any changes in basal ANP secretion may indicate that regardless of the synthesis pathway, cGMP does not participate in the basal regulation of ANP secretion.

The participation of NO in OT-induced negative chronotropic and ionotropic action was confirmed in a similar study, where L-NAME significantly inhibited OT-induced negative chronotropic and ionotropic effects (MUKADDAM-DAHER et al., 2001; ONDREJCAKOVA et al,.2009). However, the use of a nonspecific NOS blocker usually induces a negative inotropic effect on the heart cell and the heart. This is probably a consequence of the blocking of NOS1 and NOS3, since under normal conditions NOS2 expression is non-existent or too small to be considered responsible for this effect. The use of a non-specific NOS blocker (L-NAME) could demonstrate only the consequence of blocking the predominant isoform. In fact, the existence of microdomains for NOS1 and NOS3 isoforms in caveolas in the sarcolemma and sarcoplasmic reticulum, respectively, show that both isoforms can regulate the flow of Ca2+ enhanced by the activation of ß-adrenergic receptors (PATON et al., 2002) In addition, it is possible that the inhibition of Ca2+ entry through L-type Ca2+ channels mediated by the NOS1 isoform may be a probable mechanism through which NO regulates contraction and stores of intracellular Ca2+, suggesting that the NO produced by this isoform may exert a negative feedback regulation on Ca2+ influx (SEDDON et al., 2007). On the other hand, studies have shown opposite effects of the NOS1 and NOS3 isoforms on the basal activity of the heart. The authors suggested that this effect is due to the compartmentalization of these enzymes in the heart cell (BAROUCH et al., 2002). However, it is still difficult to know

precisely what effect each isoform has on baseline cardiac contractility in the face of conflicting results.

In contrast, there is evidence that NO can inhibit L-type calcium currents via cGMP (Campbell et al., 1996) and that these currents are necessary for the release of ANP under stimulated conditions (REBSAMEN et al., 1997, JIN et al., 2004). It corroborates the fact that NO can act directly by decreasing calcium current via voltage-gated membrane channels (HU et al., 1997), reinforcing the hypothesis of a negative nitrergic inotropic action on cardiomyocytes by the addition of NO donors (WAHLER; DOLLINGER, 1995; FLESCH et al., 1997).

Another possibility would be to consider the cardiac effects of ANP, which by interacting with its receptors promotes the activation of guanylate cyclase. In fact, studies suggest that ANP, in isolation, inhibits cardiac Ca2+ L-type channel activity through intracellular cGMP production and then PKG activation, since 8-bcGMP reproduced the effects of ANP in isolated rabbit heart ventricular cells (TOHSE et al., 1995). These data are suggestive that ANP and NO act by complementary effects by inhibiting myocardial contractility via cGMP and PKG activity.

Finally, we can suggest that the release of ANP from cardiomyocytes may decrease OT-stimulated cardiac activity, just as the OT-induced increase in NO would result in inhibition of ANP release, since NO may act by a negative feedback mechanism via cGMP production limiting OT-induced ANP release, thus establishing an ANP production limit. In fact, the action of ANP can be limited by NO, which can also be produced in the heart, from its NOS enzymes that are present in cardiomyocytes (BALLIGAND et al., 1995), which can modulate the action of ANP via OT, with increased NO production and consequent increase in cGMP synthesis. The increased availability of cGMP in the cytoplasm is sufficient to inhibit the release of ANP, since the administration of 8-bromine cGMP, an analogue that permeates the cell membrane, induced decreased ANP release in perfused heart isolated *in vitro* (FAVARETTO et al., 1997).

5 CONCLUSION

The present study revealed that OT stimulated the basal secretion of ANP that was inhibited by its specific antagonist, evidencing a possible mechanism of intrinsic stimulatory regulation in the cardiomyocyte.

The TO stimulated the production of NO concomitantly with that of ANP. The NO produced exhibited an inhibitory effect on basal or OT-stimulated ANP secretion. A dependent effect of cGMP synthesis via soluble guanylate cyclase. Revealing that OT can stimulate ANP secretion and NO production concomitantly. NO can activate soluble guanylate cyclase, which in turn synthesizes cGMP from GTP. Increased cGMP production may result in inhibition of ANP release by PKG activation

Finally, new experimental approaches should be conducted in order to further evaluate this relationship of dependence or regulation between these messengers and their role in the regulation of ANP in cardiomyocytes.

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