Cardiac oxytocin receptor blockade stimulates adverse cardiac remodeling in ovariectomized spontaneously hypertensive rats

Marek Jankowski, Donghao Wang, Bogdan Danalache, Marius Gangal, and Jolanta Gutkowska

Centre de recherche, Centre hospitalier de l’Université de Montréal, Hôtel-Dieu, Montréal, Québec, Canada

Submitted 28 May 2009; accepted in final form 6 April 2010

Jankowski M, Wang D, Danalache B, Gangal M, Gutkowska J.
Cardiac oxytocin receptor blockade stimulates adverse cardiac remodeling in ovariectomized spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 299: H265–H274, 2010; doi:10.1152/ajpheart.00487.2009.—An increasing amount of evidence demonstrates the beneficial role of oxytocin (OT) in the cardiovascular system. Similar actions are attributed to genistein, an isoflavonic phytoestrogen. The treatment with genistein activates the OT system in the aorta of ovariectomized (OVX) Sprague-Dawley (SD) rats. The objective of this study was to determine the effects of low doses of genistein on the OT-induced effects in rat hypertension. The hypothesis tested was that treatment of OVX spontaneously hypertensive rats (SHRs) with genistein improves heart structure and heart work through a mechanism involving the specific OT receptor (OTR). OVX SHRs or SD rats were treated with genistein (in μg/g body wt sc, 10 days) in the presence or absence of an OT antagonist (OTA) [d(CH2)5, Tyr(Me)2, Orn8]-vasotocin or a nonspecific estrogen receptor antagonist (ICI-182780). Vehicle-treated OVX rats served as controls. RT-PCR and Western blot analysis demonstrated that left ventricular (LV) OTR, downregulated by ovariectomy, increased in response to genistein. In SHRs or SD rats, this effect was blocked by OTA or ICI-182780 administration. The OTR was mainly localized in microvessels expressing the CD31 marker and colocalized with endothelial nitric oxide synthase. In SHRs, the genistein-stimulated OTR increases were associated with improved fractional shortening, decreased blood pressure (12 mmHg), decreased heart weight-to-body weight ratio, decreased fibrosis, and lowered brain natriuretic peptide in the LV. The prominent finding of the study is the detrimental effect of OTA treatment on the LV of SHRs. OTA treatment of OVX SHRs resulted in a dramatic worsening of ejection fractions and an augmented fibrosis. In conclusion, these results demonstrate that cardiac OTRs are involved in the regulation of cardiac function of OVX SHRs. The decreases of OTRs may contribute to cardiac pathology following menopause.

oxytocin receptor; ovariectomy; hypertrophy; genistein

THE BIOLOGICAL ACTIVITIES of oxytocin (OT) are associated with reproductive functions, such as uterine contraction, milk ejection, and maternal care. More recently, OT has been found to regulate vascular tone, blood pressure (BP), and kidney function (16). The pathophysiological roles of OT action in the cardiovascular system are beginning to be understood. The heart is a site of OT synthesis and action (15, 21, 22). In the adult rat heart, OT receptors (OTRs) are mainly expressed in endothelial cells and in cardiomyocytes (21). OTR activation in the heart stimulates cardioprotective reactions, such as negative inotropy and chronotropy, parasympathetic neuromodulation, and the release of atrial natriuretic peptide (ANP) (15) and nitric oxide (NO) (33). OTR-specific signaling induces the differentiation of embryonic stem cells that have been shown to convert to cardiac muscle cells (8, 21, 35). Recently, we observed that OT increases glucose uptake in cardiomyocytes via the cardioprotective phosphoinositide 3-kinase (PI3K) pathway and potentiates the glucose uptake effect of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation targeting the mitochondria (11).

The increased OTRs in the atria and ventricles of ovariectomized (OVX) rats was reported as an effect of endurance training accomplished by the reduction of cardiac hypertrophy and by beneficial outcomes on body composition (17). This study documented that in Sprague-Dawley (SD) rats, ovariectomy reduced OTRs in the left ventricle (LV), as well as also reducing the influence of physical forces on OTRs, ANP, and NO synthase expression (17). We also showed increased OTRs and endothelial NO synthase (eNOS) in the aortic tunica intima of OVX rats treated for 10 days with 17β-estradiol or genistein, the phytoestrogen (42). Genistein has an affinity to estrogen receptors (ERs)-α and -β and is considered to be the best natural ligand for ER-β (3). In the aorta of OVX SD rats, the OTR and eNOS enhancement by genistein was inhibited by nonspecific ER blockade with ICI-182780, a nonspecific ER antagonist. OT expression in the hypothalamus is downregulated in spontaneously hypertensive rats (SHRs) (12, 30, 32, 40). In this rat model, ovariectomy caused adverse cardiac and aortic wall remodeling, including cardiomyocyte hypertrophy, myocardial interstitial reparative fibrosis, and vascularization impairment with loss of cardiomyocytes (29).

The purpose of the study was to show that the treatment of OVX SHRs with genistein improves heart structure and heart echocardiographic parameters. We expected that the activated local OTR would contribute to the beneficial effects of genistein on the heart and that for this reason, the coadministration of OT antagonist (OTA) with genistein would abolish the positive effects of this phytoestrogen, such as the inhibition of cardiomyocyte hypertrophy, the reduction of collagen deposits, as well as changes in the expression profile of cardioprotective hormones.

MATERIALS AND METHODS

Animal experiments. The present experiments were conducted in accordance with the Guidelines of the Canadian Council on Animal Care and with the approval of the Animal Care Committee of the Centre hospitalier de l’Université de Montréal. OVX and sham-operated female SHRs (13 wk old, 180–190 g) and age-matched female SD rats (250–300 g) from Charles River (St. Constant, PQ, Canada) were housed individually under a 12-h:12-h light-dark cycle starting at 6:00 AM, with a room temperature of 20–23°C. The normotensive SD rats were included to provide a wider basis for baseline findings, as Wistar-Kyoto rats are not always a suitable control for SHRs because of genetic variations (26).
Genistein (Sigma, St. Louis, MO) was administered subcutaneously daily for 10 days to OVX SHR rats, starting 2 wk after ovariectomy (42). The control OVX rats were injected subcutaneously with vehicle (dimethyl sulfoxide). Genistein was administered to rats allocated to the following groups: 1) OVX rats receiving genistein (1 μg/g in 200 μl of vehicle), 2) OVX rats receiving genistein (1 μg/g in 200 μl of vehicle), 3) OVX rats receiving genistein with the ER antagonist ICI-182780 (1.5 μg·g⁻¹·day⁻¹) sc for 10 days; Tocris, Ellisville, MO), 4) OVX rats receiving both genistein and OTA [d(CH2)5, Tyr(Me)2, Orn8]-vasotocin (0.1 μg/g body wt; Peninsula, Belmont, CA), and 5) OVX rats receiving only OTA in 200 μl of vehicle. The rats were housed in pairs in a light-controlled room (12:12-h light-dark cycle) with free access to tap water and placed on a soybean-free diet (AIN 76). Body weight (BW) was measured every 2 days. Systolic BP and heart rate (HR) were recorded by the tail-cuff method with the Visitech BP-2000 system (Apx, NC) after 3 days of training before injection and on the last day of the experiment from 9:00 AM to 11:00 AM daily. At the end of the experiment, the rats were decapitated, and their hearts were rapidly removed and blotted dry. The heart weight was recorded (Mettler AE 100) after the dissection of the left and right atria, right ventricle, and LV with septum, followed by an immediate freezing in liquid nitrogen and storage at -80°C. Blood was centrifuged (3,000 rpm at 4°C, for 10 min), and plasma was kept at 80°C. Blood was centrifuged at 30,000 g for further analysis. Total heart and ventricle weight-to-BW ratios expressed the degree of myocardial hypertrophy. Plasma estrogen levels and the absence of the ovaries and uterine ventricle weight-to-BW ratios expressed the degree of myocardial hyper- and plasma was kept at storage at 80°C. Blood was centrifuged (3,000 rpm at 4°C, for 10 min), and plasma was kept at 80°C for further analysis. Total heart and ventricle weight-to-BW ratios expressed the degree of myocardial hypertrophy. Plasma estrogen levels and the absence of the ovaries and uterine ovary atrophy at necropsy verified the adequacy of ovariectomy. Bilateral ovariectomy produced plasma 17β-estradiol levels of 5.7 ± 1.0 pg/ml.

Radioimmunoassay. To quantify ANP in the plasma, 2 ml of blood were collected in chilled tubes containing 1 mg/ml EDTA, 10 mM PMSF (P7626; Sigma), and 5 mM pepstatin A (P4265; Sigma). After centrifugation for 20 min at 4°C at 4,000 rpm, the separated plasma was extracted immediately. Plasma ANP was measured by radioimmunoassay (RIA) after extraction in C18 Sep-Pak cartridges (Waters Chromatography Division, Millipore, Milford, MA) as described elsewhere (14). To quantify tissue ANP and brain natriuretic peptide (BNP) concentrations, the LVs were homogenized in 0.1 M acetic acid containing protease inhibitors to a final concentration of 1 mg/ml of EDTA, 5 μM peptatin A, and 10 μM PMSF at 4°C in a Polytron homogenizer (setting, 10 × 3 for 20 s). After 20 min of centrifugation at 30,000 g, the pellet was washed and rehomogenized in the same buffer. The ANP concentration was measured by direct RIA in serial dilutions of the homogenates (14). The values were normalized to protein concentration (reported as μg/mg protein).

Plasma OT was quantified according to a previously published method (22).

Reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from frozen samples with TRIzol (Invitrogen Life Technologies, Burlington, ON, Canada), according to the manufacturer’s protocol. To remove genomic DNA, RNA samples were incubated with 2 units deoxyribonuclease I (DNase I; Invitrogen Life Technologies)/μg RNA for 30 min at 37°C. Semiquantitative PCR was performed to compare OTR and ER mRNA expression in the different groups. The sequences of specific primers are presented in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer (5’-3’)</th>
<th>Antisense Primer (5’-3’)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTR</td>
<td>GTCATCGGCAGCAAGAGAAG</td>
<td>GATGCAAAACCAATAGACACC</td>
<td>NM_012871</td>
</tr>
<tr>
<td>ER-α</td>
<td>ATCTGGAGATCAAGACGCAG</td>
<td>GTGCTTCACATTTCCCTCCTC</td>
<td>NM_012689</td>
</tr>
<tr>
<td>ER-β</td>
<td>TCTCAGGTGCGCTCTATCA</td>
<td>TCTGTCTTGGCTTGGACTA</td>
<td>NM_012754</td>
</tr>
<tr>
<td>ANP</td>
<td>GACATGACCATTTCCCTCCT</td>
<td>GTCGATCTAAGCCGAAAGCT</td>
<td>NM_012612</td>
</tr>
<tr>
<td>BNP</td>
<td>CCCATGAGCTTCCCTGCGATC</td>
<td>GACATGCGGATCCTGC</td>
<td>NM_031543</td>
</tr>
<tr>
<td>OT</td>
<td>AGGCGAGCTGACGTGGAGA</td>
<td>CGCCGCTTAAAGGTACCTC</td>
<td>NM_02996</td>
</tr>
</tbody>
</table>

OTR, oxytocin (OT) receptor; ER-α, estrogen receptor-α; ER-β, estrogen receptor-β; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

A volume of 5 μl first-strand cDNA was added to a PCR mixture and amplified for 30–35 cycles by incubation at 95°C for 30 s, at 65°C for 30 s, and at 72°C for 45 s, with a final incubation at 72°C for 5 min, all in a Robocycler gradient 40 thermocycler (Stratagene, La Jolla, CA). The specificity of the amplified products was verified by sequencing. Amplification of 18S RNA, as an internal standard, followed the manufacturer’s protocol (Ambion, Austin, TX). The PCR products, fractionated onto 1.2% agarose gels containing ethidium bromide, were size fractionated by 2% agarose gel electrophoresis and quantified with the Storm 840 imaging system and ImageQuant software (version 4.2; GE Healthcare, Sunnyvale, CA). To validate this RT-PCR assay as a tool for the semiquantitative assessment of mRNA, dose-response curves were charted for different amounts of total RNA extracted from the rat LV, and the samples were quantified in the curvilinear phase of PCR amplification. The expression levels were normalized against either 18S RNA or GAPDH and presented in arbitrary units versus the mean of control group estimated as 100. No difference in 18S RNA or GAPDH levels was observed in any experimental group.

Western blot analysis. Western blot analysis was conducted as described elsewhere (17). Blots were incubated overnight at 4°C with mouse monoclonal antibodies for ER-α and rabbit polyclonal antibodies for ER-β (BD Biosciences, San Jose, CA). OTRs were investigated with goat anti-OTR antibody obtained from Santa Cruz Bio- technology (sc-8102, Santa Cruz, CA, http://www.scbt.com), followed by an incubation with peroxidase-conjugated secondary antibody (GE Healthcare). Proteins were visualized with an Amer sham ECLT GST Western Blotting Detection Kit (GE Healthcare). Densitometry was undertaken with the Scion program (National Institutes of Health, Bethesda, MD).

Echocardiography. Transthoracic echocardiographic studies were performed on rats anesthetized with 1.5% isoflurane supplemented with O2. Their hearts were investigated in a vivid GE ultrasound machine with a 10-MHZ transducer that provided M-mode tracings. M-mode tracings were recorded according to American Society of Echocardiography guidelines. M-mode images from both a short and long axis were analyzed to determine LV end-diastolic/systolic dimension and shortening fractions. The following formula was used to calculate LV fractional shortening: percent fractional shortening = (end-diastolic diameter - end-systolic diameter)/end-diastolic diameter × 100. The ejection fraction was obtained from the short-axis view and calculated according to the following formula: diastolic area - systolic area/diastolic area. Images were analyzed with ImageJ software (http://rsb.info.nih.gov/ij/).

Histology. The hearts were fixed by perfusion with 4% formaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4), embedded in wax and cut into 5-μm sections. Histological examination was conducted in sections from the cardiac intraventricular septum by hematoxylin-phloxine-safranine and Masson trichrome staining. Photographs were taken with a Nikon Eclipse Model T Square 2000-S-inverted microscope (Nikon, Tokyo, Japan, http://www.nikon.com) equipped with a Q Imaging QICAM-IR Fast Digital 1394 charge-coupled device camera. Cells were measured by Micro Dimension Version 1.01 analysis software. The perimeters of at least
10 cells in seven photographs were examined manually and calculated in squared micrometers. In Masson trichrome-stained sections, collagen areas were quantified with ImageJ software. The interstitial collagen volume fraction was measured while omitting fibrosis of the perivascular, epicardial, and endocardial areas. The collagen volume fraction was obtained by calculating the mean ratio of connective tissue to the total tissue area of all measurements of the section. The collagen-positive areas from all sections were assessed by a single investigator who was unaware of the experimental groups.

**Immunocytochemistry.** Staining was performed as reported elsewhere (42). OTR were detected by a goat antibody (sc-8102, Santa Cruz Biotechnology), and a goat anti-CD31 (platelet endothelial cell adhesion molecule 1, sc-1506, Santa Cruz Biotechnology) antibody was used to show blood vessel density. Immune complexes were quantified with ImageJ software. The interstitial collagen volume fraction was measured while omitting fibrosis of the perivascular, epi-, and endocardial areas. The collagen volume fraction was obtained by calculating the mean ratio of connective tissue to the total tissue area of all measurements of the section. The collagen-positive areas from all sections were assessed by a single investigator who was unaware of the experimental groups.

**Data analysis.** Data are presented as means ± SE. Means were compared by one-way ANOVA followed by Tukey’s posttests. Two-way ANOVA was undertaken to analyze the data with status (sham operation and ovariecomy) and strain (SD and SHRs) as independent factors and plasma and receptor levels as dependent variables. Similar two-way analysis was performed to investigate the effects of genistein, OTA, and their combinations. P < 0.05 was considered statistically significant.

![Fig. 1. A and B, top: effect of genistein treatment of ovariecomized (OVX) Sprague-Dawley (SD) rats on gene expression in the left ventricle (LV; A) and right atrium (B). C, top: oxytocin (OT) plasma level (C1) and OT expression in the hypothalamus (C2). Genistein treatment enhanced OT receptor (OTR) mRNA (A1 and B1) and estrogen receptor (ER)-α mRNA (A2 and B2) and increased ER-β mRNA in the LV (A3) but not in right atria (B3) of OVX SD rats. Analysis of RT-PCR amplification using specific oligonucleotide primers in the specimens of sham-operated rats (sham), OVX rats treated with vehicle (V), and OVX rats treated with 1 μg·g−1 day−1 genistein (G), OVX rats treated with genistein plus OT antagonist (G + OTA), and OVX rats treated with genistein plus ICI-182780 (G + ICI) is shown. Bars represent means ± SE for samples derived from 8–10 rats in each treatment group. A–C, bottom: representative bands of PCR amplification products vs. molecular weight DNA ladder. In A1–A3 and C2, the 3 representative bands were excised from the gel scan and placed under the corresponding graph bars. &P < 0.05, significant difference between sham and OVX (V) rats; *P < 0.05, significant effect of genistein treatment in the presence or absence of antagonists vs. OVX (V) group; †P < 0.05, significant effect of antagonist (OTA or ICI) on genistein treatment vs. OVX (G) group. All y-axis values are in arbitrary units, except where indicated otherwise.**
RESULTS

Effect of treatments on physiological parameters of SHRs. Table 2 reports the effect of genistein treatment on BW, heart weight, heart-weight-to-BW ratio, uterine weight, BP, and HR. Genistein treatment of OVX SHRs decreased BP (12 mmHg lower than in vehicle-treated OVX SHRs, \( P < 0.05 \)) without significant changes in HR. This effect was absent in rats receiving genistein combined with OTA or ICI-182780. Vehicle-treated OVX SHRs gained an average of 34 g, 18% of initial BW, and genistein alone or in combination with OTA or ICI-182780 did not influence BW. In addition, genistein treatment did not significantly change weight. Genistein-induced decreases in heart weight-to-BW and LV weight-to-BW ratios in OVX SHRs were reverted by ICI-182780 supplementation.

Effect of genistein treatment on OT and natriuretic peptide systems in OVX SD rats. As presented in Fig. 1, A1–A3, ovariectomy and genistein treatment modulated the mRNA level of OTR and both ERs in the LV of SD rats (\( P < 0.05 \)). Ovariectomy reduced the expression of OTR (Fig. 1A1), ER-\( \alpha \) (Fig. 1A2), and ER-\( \beta \) (Fig. 1A3) transcripts. In OVX rats, genistein treatment counteracted the effect of estrogen deficiency and resulted in potent (3-fold) stimulation of OTR gene expression in the LV (Fig. 1A1). This change was associated with the significant (2-fold) enhancement of ER-\( \alpha \) (Fig. 1A2) and ER-\( \beta \) (Fig. 1A3) mRNA. The genistein effects on OT gene expression in the LV of SD rats were blocked by simultaneous treatment with OTA or by ICI-182780. Treatment with OTA + genistein did not alter changes in the expression of ER-\( \alpha \) and ER-\( \beta \) mRNA exerted by genistein. The stimulation of ER-\( \beta \) mRNA by genistein treatment was prevented by ICI-182780 administration.

Similarly, as in the LV, genistein treatment increased (6-fold) OTR mRNA (Fig. 1B1) and increased ER-\( \alpha \) mRNA expression (Fig. 1B2) (\( P < 0.05 \)). OTA administrated with genistein reverted the expression of OTR mRNA to the level observed in OVX rats treated with vehicle but had no effect on the genistein-induced reduction of ER-\( \alpha \) mRNA. ICI-182780 supplementation inhibited genistein-induced OTR mRNA and ER-\( \alpha \) mRNA. Genistein treatment in the presence or absence of both antagonists had no effect on ER-\( \beta \) mRNA expression by genistein treatment was prevented by ICI-182780 administration.

Low OT concentration in plasma of OVX SD rats increased after genistein treatment (Fig. 1C1). In rats simultaneously receiving genistein with OTA, the plasma OT level was reduced but still significantly higher than in OVX SD control rats. Similarly, in the rats receiving genistein with ICI-182780, the plasma OT level tended to be lower compared with rats receiving genistein alone (\( P = 0.08 \)). OT mRNA in the hypothalamus increased significantly (2.6-fold) in OVX rats treated with genistein, and this increase was lowered by the administration of OTA or ICI-182780 (Fig. 1C2). Interestingly, the OT plasma level paralleled the changes in OT mRNA expression induced in the hypothalamus by treatment with genistein in the presence and absence of antagonists.

Effect of ovariectomy on the OT system and ERs in the LV of SD rats and SHRs. We compared the effect of ovariectomy in SHRs and SD rats on the plasma OT level and OTR and on the ER-\( \alpha \) and ER-\( \beta \) expression in the LV. Figure 2A shows that plasma OT concentration in OVX rats was lower than in sham-operated animals (\( P = 0.006 \)). Post hoc analysis indicated that ovariectomy significantly downregulated OT plasma concentration in SD rats, but not in SHRs. Western blot analysis of OTR and ER proteins in the LV revealed that ovariectomy downregulated OTR (\( P = 0.0045 \)), ER-\( \alpha \) (\( P = 0.001 \)), and ER-\( \beta \) (\( P = 0.006 \)) (Fig. 2, B–D). However, a post hoc testing disclosed that OTR and ER-\( \alpha \) were lowered in both rat strains, but ER-\( \beta \) decreased after ovariectomy of SHRs and not SD rats. Because similar data were obtained by RT-PCR analysis, it can be concluded that OTR and ERs are lowered by ovariectomy at the transcription level (Fig. 2, B–D). A rat strain effect was observed only for OTR (\( P = 0.049 \)) and OTR mRNA (\( P = 0.001 \)), which indicated lower OTR expression in SHRs than in SD rats.

Effect of genistein on OTR expression in the LV of SHRs. Western blot analysis showed that genistein treatment significantly augmented OTR in the LV of OVX SHRs (\( P = 0.001 \)), and this effect was reduced by simultaneous treatment with OTA or ICI-182780 (Fig. 2A). Immunocytochemistry demonstrated that compared with vehicle treatment (Fig. 3B1), genistein treatment (Fig. 3B2) stimulated OTR in cardiac structures. In a similar way, immunostaining of endothelial CD31 was enhanced in cardiac sections from genistein-treated
animals compared with vehicle-treated controls (Fig. 3, C1 vs. C2). When comparing sections from animals treated with genistein alone, the OTR and CD31 staining were reduced in sections collected from animals simultaneously treated with genistein and the OTA (Fig. 3, B3 and C3) or ICI-182780 (Fig. 3, C3 and C4). Further analysis performed by immunofluorescence costaining revealed the colocalization of OTR and CD31 (Fig. 3, D1–D3) as well as the colocalization of OTR and eNOS (Fig. 3, D1–D3).

**RIA of natriuretic peptides.** An analysis of ANP concentration by RIA did not disclose significant differences in plasma samples from differently treated groups of OVX SHRs (Fig. 4A). On the other hand, local ANP content in the LV was lowest in OVX SHRs treated with genistein + OTA and significantly different from those receiving vehicle or genistein alone (Fig. 4B, P = 0.02).

Because BNP is a sensitive indicator of LV alterations, we undertook RIA to monitor changes of BNP in OVX SHRs. As presented in Fig. 4C, BNP concentration decreased in the LV of genistein-treated versus vehicle-treated rats (P = 0.04). OTA combined with genistein reversed BNP in the LV to the level seen in vehicle-treated controls. Therefore, OTR blockade by a specific antagonist reduced the beneficial effect of genistein on BNP in the LV of OVX SHRs.
fractional shortening and ejection fraction (Fig. 5, OTA in the presence and absence of genistein decreased D, but treatment with improved fractional shortening (Fig. 5 indicated by echocardiography, genistein treatment in OVX SHRs alone, treated with genistein combined with OTA or ICI-

analysis indicated that cardiomyocyte size was significantly larger in rats treated with vehicle than in those treated with genistein alone, treated with genistein combined with OTA or ICI-182780, or treated with OTA alone (Fig. 6F). When compared with those of rats treated with genistein, larger cardiomyocytes were observed in rats receiving genistein combined either with OTA or ICI-182780. Low fibrotic deposits were found in cardiac sections from OVX SHRs treated with vehicle (Fig. 6G) and in rats receiving genistein (Fig. 6H). Interestingly, treatment with either OTA (Fig. 6J) or genistein combined with both antagonists induced fibrotic deposits in the heart (Fig. 6, I and K). The visual observations from images were confirmed by quantitative analysis using ImageJ software (Fig. 6L). The data suggest that the inhibition of OTR or ERs in OVX SHRs leads to the development of cardiac fibrosis.

DISCUSSION

Genistein treatment of the OVX rats stimulated OT plasma levels and OTR expression in the heart. In OVX SHRs, genistein treatment significantly reduced myocyte hypertrophy, increased microvascular networks assessed by CD31 immunohistochemistry, and enhanced fractional shortening. In the course of this study, we observed that the rats receiving genistein combined with OTA displayed a worsening of cardiac morphology and heart work at a higher extent than those expected after blocking genistein action. Further analysis of these parameters in the rats treated with OTA alone indicated that the major finding of this study is the damaging effect of OTA on the heart.

The harmful effect of OTA is consistent with very recent observations that experimental myocardial injury is associated with OTR reduction in the heart (19). We then demonstrated that OTR, initially downregulated in the infarcted heart, was subsequently activated in response to OT infusion (20). Furthermore, in rat and rabbit models of ischemic heart disease, OT treatment significantly decreased infarct size (18, 20, 23, 34). The observation that an OTA increases the severity of regional ischemic reperfusion injury in the rat indicates that the OT system is a natural cardiac defender (18).

To block OTR, we administered 0.1 μg/g body wt OTA since extensive literature indicates that this concentration can affect a variety of physiological and behavioral responses in rats and voles (4, 24, 43). The OTA proposed in this study is selective for OTR but can also bind arginine-vasopressin receptor 1, albeit at a much lower affinity (28), and has been used previously in voles with differential effects on OT and arginine-vasopressin receptors (5, 6). Our recent reports provide a partial explanation of how OT can act on the cardiac cells. The treatment with OT increases the glucose uptake in the cardiomyocytes both in normal as well as in the hypoxic conditions generated by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation in the mitochondria (11). In this study, the OT signal transduction inhibited by OTA in the cardiomyocytes included the PI3K pathway and the calcium-calmodulin kinase kinase-AMP-activated protein kinase pathways recognized as cardioprotective because both limits apoptosis and cell damage (27, 31, 38).

Cardiac OTR inhibition by OTA in OVX SHRs reduced ejection and shortening fractions, which likely were due to decreased cardiac contractility. However, OTA treatment induced a relatively modest effect on systolic BP and LV weight but a dramatic functional effect on the LV echocardiographic

![Figure 4](image-url)
Evidence for the use of genistein as a cardioprotective estrogen

Mediated decline of BP attenuates cardiac hypertrophy via the OTR pathway. In the same model, the PI3K/Akt signaling was upregulated in the cardiovascular system also involves ER-α. Based on this observation, we concluded that OTR upregulation in the cardiovascular system also involves ER-α activation. This raised the doubt that a pure ER-β agonist will not be effective in the activation of the cardiac OTR. An important hint was also provided by reports stating that in knockout mice, ER-α is not necessary for basal OTR synthesis but is essential for the induction of ligand binding to OTR (44).

It already has been reported that genistein lowered elevated BP and endothelial dysfunction in SHRs in relation to augmented ENOS activity in the aorta (41). Possibly, the genistein-mediated decline of BP attenuates cardiac hypertrophy via parameters. This effect of OTA may involve neuronal regulation. In recent experiments on the isolated, perfused hearts of Wistar rats subjected to ischemia-reperfusion injury, the administration OT resulted in a negative chronotropy and a reduction of infarct size (34). The elimination of the negative chronotropic effect of OT prevented cardioprotective action. On the basis of our previous data (33), the authors hypothesized that OT, by activating intrinsic cardiac cholinergic neurons and NO release, can effectively inhibit cardiac sympathetically mediated decrease in LV ejection fraction in rats with myocardial injury.

It was also reported that a relatively short period of time of treatment (2 days) was associated with significant anti-ischemic cardioprotective effects of genistein in OVX rats (2). Evidence for the use of genistein as a cardioprotective estrogen comes from the study by Tissier et al. (39), in which genistein (100 μg/kg) was found to exert pharmacological preconditioning, using a rabbit model of myocardial infarction, via an estrogen receptor-dependent mechanism, using the PI3K/Akt pathway. In the same model, the PI3K/Akt signaling was stimulated by OT treatment (23).

Like other phytoestrogens, genistein can work in two ways: either by increasing or decreasing the effects of estrogen. This happens because genistein combines to ER. Genistein regulates these receptors, and at the same time, it blocks estrogen binding to ER. For this reason, in this study the OVX rats and not sham-operated rats were treated with genistein. Indeed, the reductions of the genistein effects on BP, cardiac fibrosis, and BNP concentration in the LV of OVX SHRs by the nonselective ER antagonist ICI-182780 suggest that an estrogenic mechanism is involved in the action of genistein. In this and in a previous study (42), we did not investigate higher doses of genistein than 1 μg/g because they are well known to induce tyrosine kinases inhibition (1), which was proposed to alter the protection induced through estrogenic activation (10). In higher doses, genistein improved heart function not as an estrogen but via other mechanisms, such as through the activation of calcium channels (13), inhibition of tyrosine kinases (1), which was proposed to alter the protection induced through estrogenic activation (10). In higher doses, genistein improved heart function not as an estrogen but via other mechanisms, such as through the activation of calcium channels (13), inhibition of tyrosine kinases (1), which was proposed to alter the protection induced through estrogenic activation (10).
cardiac afterload diminution, because fibrotic deposits are unchanged. However, the increase of BP seen in SHRs may reflect microvascular dysfunction, and it is possible that, to inhibit hypertension, genistein targets ER-β expressed in resistance arteries (7). Interestingly, microvascular networks assessed by CD31 immunohistochemistry appeared to be defective within the group receiving vehicle but activated in the genistein-treated group of OVX SHRs. This mechanism also enhanced OTR, colocalized with eNOS in cardiac microvessels, as reported in this study. The beneficial effect of genistein on the rabbit cardiac microvessel network in ischemic conditions has already been reported (39).

In summary, these data demonstrate that genistein treatment of OVX SHRs activates OTR in cardiac microvessels, reduces BP, and improves fibrosis in the LV. OTR importance in these rats is indicated by the detrimental outcomes of OTA treatment on the LV. A new finding is that the OT system in SHRs can contribute significantly in the control of cardiac function in hypertension and may be involved in cardiovascular pathologies arising after menopause.

Fig. 6. Representative histopathological images of the heart from treated OVX SHRs. Hematoxylin-phloxine-safranine staining shows cardiomyocyte enlargement in rats treated with V (A) compared with cardiomyocytes in sections from rats treated with G (B). Cardiomyocytes from rats treated with G + OTA (C), OTA (D), or G + ICI (E) were significantly larger than those from rats treated with G alone. The results were verified by calculating of cardiomyocyte size with ImageJ software (F). Masson trichrome staining (G–K) shows low collagen deposits (blue) in sections of the heart from rats treated with V (G) or G (H). Abundant collagen deposition is detected in sections from rats treated with G + OTA (I), OTA (J), or G + ICI (K). Quantification of collagen deposits after genistein treatment vs. vehicle control did not give significant difference. Cells in the sections are stained in red. L: quantification of collagen deposits expressed as blue-to-red ratios. For each sample (n = 4 in each group), 7 fields were counted. Significant differences: *P < 0.05 from vehicle, and &P < 0.05 from genistein. Bar = 50 μm.
ACKNOWLEDGMENTS

The editorial assistance of Ovid Da Silva, Centre de recherche du CHUM-Hôtel-Dieu, is acknowledged.

GRANTS

This work was supported by the Canadian Heart and Stroke Foundation NET Program Grant SRD-63193 (to J. Gutkowska and M. Jankowski) and Canadian Institutes of Health Research NET Program Grants MT-15049 and MOP-18793 (to J. Gutkowska and M. Jankowski).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


2. Al-Nakkash L, Markus B, Bowden K, Batia LM, Prozialeck WC, Rice-Evans CA. Free radical scavenging and nitric oxide signaling by 10.220.32.247 on October 6, 2016 http://ajpheart.physiology.org/ Downloaded from


