Genistein supplementation stimulates the oxytocin system in the aorta of ovariectomized rats

Donghao Wang, Jolanta Gutkowska, Mieczyslaw Marcinkiewicz, Grazyna Rachelska, Marek Jankowski

Abstract

Objective: In the present study, we localized oxytocin (OT) and its receptor (OTR) in the rat aorta, and investigated whether genistein, an isoflavonic phytoestrogen, influences their expression in ovariectomized (OVX) rats deficient in estrogen. Methods and results: OVX Sprague-Dawley rats were randomized to the following groups: genistein (from 0.02 to 5 μg/g/day, s.c. for 10 days), estradiol (E2 0.1 μg/g/day, s.c. for 10 days) or their respective vehicles. OT and OTR immunostaining was concentrated in the aortic tunica intima, suggesting their paracrine/autocrine action within endothelial cells. Reverse transcription-polymerase chain reaction analysis showed that 1 and 5 μg/g but not 0.1 μg/g genistein elevated OT mRNA (2-fold, P<0.05), OTR mRNA (2.5-fold, P<0.05) and endothelial nitric oxide synthase (eNOS) mRNA (2-fold, P<0.05) in the aorta of OVX rats. In addition, genistein treatment increased estrogen receptor α (ERα) (2- to 3-fold, P<0.05) but resulted in a 50% decrease of ERβ (P<0.05). These genistein effects were neutralized by treatment of OVX rats with the ER antagonist ICI 182,780 (1.5 μg/g/day, s.c. for 10 days). Similarly, Western blot analysis revealed an increase of 67-kDa OTR, 140-kDa eNOS, 62-kDa ERα and a decrease of 55-kDa ERβ (P<0.05) in the aorta of OVX rats treated with genistein. In contrast, the treatment of OVX rats with E2 elevated ERβ mRNA (1.5 fold, P<0.05) but similarly to genistein increased OT, OTR, eNOS and ERα mRNA. Conclusion: These results provide the first evidence of OT and OTR co-localization in endothelial cells. The response to genistein via ER activation can be regarded as a recovery from endothelial dysfunction induced by ovariectomy.

Keywords: Endothelial function; Gene expression; Hormones; Receptors; Vasoconstriction/dilation

1. Introduction

Oxytocin (OT), a neurophyseal hormone, is known to play a role in lactation and parturition and to act in the central nervous system as a neurotransmitter involved in sexual and maternal behaviour. OT and its receptor (OTR) have been identified in a variety of peripheral tissues of both genders, suggesting that OT has other than maternal physiological functions [1]. OT has been discovered in the aorta, vena cava and heart. In the rat, it acts in concert with atrial natriuretic peptide (ANP) in the control of volume homeostasis [2–4], and OTR stimulation induces ANP release in vitro from isolated, perfused rat hearts [4]. Vascular OTR is structurally identical to uterine OTR [5]. Together with previous evidence of OT and OTR expression in the heart [6], the demonstration of widespread OT binding sites in the rat vasculature and of OT synthesis at that site [5] has pointed to its autocrine and paracrine roles in cardiovascular function.

Available data suggest that vascular OT is implicated in volume and blood pressure (BP) regulation. Indeed, localization of the OTR gene in proximity to the quantitative...
trait locus for BP in Dahl salt-sensitive rats supports the concept of OT involvement in BP regulation [7]. This vascular OT effect may involve both cGMP [8] and nitric oxide (NO) release [9] from vascular endothelial cells. OT also acts as a neurotransmitter in the heart and induces negative chronotropic and inotropic effects in isolated dog atria, an effect mediated by intrinsic cholinergic neurons and involving NO signaling [10].

17β-Estradiol (E$_2$) stimulates NO from endothelial cells, but the mechanisms involved are unclear and under debate. Estrogens exert direct effects on the blood vessel wall by enhancing NO bioavailability through stimulation of endothelial nitric oxide synthase (eNOS) and subsequent NO release [11]. In contrast, decreased NO bioavailability leads to endothelial dysfunction. Ovariectomized (OVX) rats, which are deficient in estrogen, display endothelial dysfunction [12], which can be reversed by chronic treatments with E$_2$ or genistein, an isoflavonoid phytoestrogen, indicating modulation of vasodilation via changes in eNOS gene expression [12].

Genistein present in soy products, a traditional Asian diet, has been associated with health benefits, such as the prevention of hormone-dependent coronary heart disease. The cardioprotective ability of these plant products has been attributed, at least in part, to their ability to decrease cholesterol concentration in the circulation [13], to lower BP [14] and to improve arterial compliance [15]. Chronic treatments with both genistein and E$_2$ also provide vasoprotection in a rat model of carotid injury [16]. Other genistein effects include inhibition of vascular smooth muscle cell (SMC) replication [17], and enhancement of the vascular reactivity to acetylcholine in atherosclerotic arteries [18]. The relaxation of agonist-constricted arteries by genistein is endothelium-dependent [19] and involves NO [20].

In the present study, we tested the hypothesis that genistein acts via estrogen receptors (ER), and up-regulates the endothelial OT system in a manner similar to E$_2$ in the rat aorta. Because of the multifactorial nature of vascular function and numerous mechanisms by which genistein could influence vascular cells, we investigated its dose-dependent actions on the OT system and eNOS expression in the aorta of OVX rats. We also assessed the effects of genistein treatment on body weight, uterus weight, and the expression of aortic ERα and ERβ.

2. Methods

2.1. Animals

These experiments were conducted in accordance with the Guidelines of the Canadian Council on Animal Care with the approval of the committee of the Centre Hospitalier de l’Université de Montréal. OVX, Sham-operated Sprague–Dawley rats (200–220 g) were obtained from Charles River (Ste-Foy, Quebec, Canada). Both E$_2$ and genistein (Sigma, St. Louis, MO) were administered (s.c.) daily for 10 days to OVX rats, starting 2 weeks after surgery. The doses for genistein and E$_2$ were selected on the basis of investigations by Mäkelä et al. [16] who demonstrated the vasculoprotective effect of these compounds in the OVX rat carotid injury model. Genistein was administered to rats allocated to the following groups: (1) group of sham-operated controls receiving 200 µl of vehicle (98.75% PEG-400+1.25% DMSO, n=20); (2) group of OVX controls receiving 200 µl of vehicle (n=20); (3) groups of OVX rats receiving genistein (0.02 (n=10), 0.1 (n=10), 1 (n=20) or 5 µg/g (n=20)) in 200 µl of vehicle. Ten OVX rats receiving genistein were treated also with the ER antagonist ICI 182,780 (1.5 µg/g/day, s.c. for 10 days, purchased from Tocris, Ellisville, MO). Other rats were randomly allocated to one of three groups: (4) group of sham-operated controls receiving 100 µl of sunflower oil (vehicle, n=10); (5) group of OVX rats receiving 100 µl of vehicle (n=10); and (6) group of OVX rats receiving 0.1 µg/g of E$_2$ in 100 µl of vehicle (n=10). Rats were housed in pairs in a light-controlled room (12-h light/dark cycle) with free access to tap water and placed on a soybean-free diet. Body weights were recorded at the time of ovariectomy, and every 2 days over 10 days of treatment. Body weight gain was calculated for each animal and averaged for each group. The rats were killed by decapitation at the end of the experiment. The thoracic aorta and uterus were rapidly removed, dissected from connective tissue, weighed, and frozen in liquid nitrogen. Tissues were stored at −80°C until processed. E$_2$ and progesterone levels were measured in a Advia Centaur automated chemiluminescence system (Bayer, Toronto, Ontario, Canada).

2.2. Physiologic measurements

The adequacy of ovariectomy was verified at necropsy by the absence of ovaries, uterine atrophy, and low plasma E$_2$ levels. Systolic BP and heart rate (HR) were recorded by the tail-cuff method with the Visitech BP-2000 system (Apex, NC) after 3 days of rat training before treatment and on the last days of the experiment from 09:00 to 11:00 h.

2.3. Immunocytochemistry

Immunocytochemical stainings were performed with the biotin–streptavidin method (Histostain-Plus kit, Zymed Laboratories, San Francisco, CA). OT and OTR were investigated with rabbit polyclonal antibody (generously gifted by Dr Marianna Morris, Wright State University, Dayton, OH, and Dr Kate Whittington, University of Bristol, UK, respectively). The immunoreactions were revealed by horseradish-peroxidase activity producing brown staining. Immunoreactivity was blocked by preincu-
bation of antibodies with an excess of antigen (10⁻⁶ M) in the case of OT, or by omission of the first antibody in the case of OTR, and interaction with normal goat serum.

2.4. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the aorta using Trizol reagent (Life Technologies, Rockville, MD), and RT-PCR was performed as described previously [21]. cDNA was subjected to PCR amplification, using primers for ERα, ERβ, OT, OTR, and eNOS (Table 1).

Amplification of 18S RNA by oligonucleotides followed the manufacturer’s protocol (Ambion, Austin, TX). Control RT-PCRs omitted reverse transcriptase or RNA from clonal antibodies for ERα and rabbit polyclonal antibodies for ERβ and eNOS (BD Biosciences, San Jose, CA). OTR was investigated with rabbit polyclonal antibodies (generously gifted by Dr. Kate Whittington, University of Bristol, UK, respectively). The membranes were washed and incubated with secondary antibodies linked to horseradish peroxidase labeled antimouse or antirabbit IgG (Amersham Pharmacia Biotech) for 2 h. The blots were developed in an enhanced chemiluminescence system (Amersham Pharmacia Biotech) for 2 h. The data are expressed as means±S.E.M. Normalized amounts of total RNA extracted from the rat aorta, and eNOS (BD Biosciences, San Jose, CA). OTR was investigated with rabbit polyclonal antibodies (generously gifted by Dr. Kate Whittington, University of Bristol, UK, respectively). The membranes were washed and incubated with secondary antibodies linked to horseradish peroxidase labeled antimouse or antirabbit IgG (Amersham Pharmacia Biotech) for 2 h. The blots were developed in an enhanced chemiluminescence system (Amersham Pharmacia Biotech) and then visualized by exposure to Kodak X-ray film. The accuracy of protein loading on the gel was verified by re-probing with rabbit β-actin antibody and protein coloration on membranes. Densitometry was done with the Scion program (NIH, Bethesda, MD).

2.5. Western blot analysis

Western blot analysis was conducted as described elsewhere [21]. Briefly, tissue samples were homogenized in sucrose buffer (0.8 ml/100 mg tissue) comprised of 20 mM Hapes/Tris with 250 mM sucrose, pH 7.4. The samples were centrifuged at 3000×g for 10 min at 4°C to eliminate cellular debris. The supernatant was removed and centrifuged at 37,500×g for 30 min at 4°C. The membrane pellets were resuspended in sucrose buffer and the protein was determined by Bradford assay. Protein was separated through −8% sodium dodecyl sulfate (SDS) polyacrylamide gel and electrotransferred to nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech, Baie d’Urfé, Canada). Unbound sites were blocked overnight at 4°C with 10% (w/v) nonfat milk in Tris-buffered saline containing 20 mmol/l Tris–HCl (pH 8.0), 140 mmol/l NaCl, and 0.05% (w/v) Tween-20. The membranes were then probed either with mouse monoclonal antibodies for ERα and rabbit polyclonal antibodies for ERβ and eNOS (BD Biosciences, San Jose, CA).

2.6. Statistical analysis

The data are expressed as means±S.E.M. Normalized data from the genistein- and E₂-treated groups were compared with their respective control groups by ANOVA, followed by Dunnett’s test, or, in case of comparison with...
3. Results

3.1. Effects of ovariectomy and genistein supplementation

Effect of genistein treatment on body weight, uterine weight, blood pressure (BP), heart rate (HR) and serum concentrations of estradiol and progesterone is presented on Table 2. Genistein treatment did not alter the decreases in plasma estradiol and progesterone levels (<3.7 pmol/l and 11.2±2.6 nmol/l, respectively) produced by bilateral ovariectomy. BP and HR values in OVX rats treated with genistein were similar to those in vehicle-treated controls. OVX animals gained an average of 43.1±2.5 g, which was greater than for sham-operated controls (31.8±3.6 g, P<0.05). Ovariectomy and treatment with 1 μg/g genistein increased body weight by 34.1±2.3 g (P>0.05 vs. OVX), but the dose of 5 μg/g significantly inhibited the body weight gain (29.9±1.7 g, P<0.01 vs. OVX). In addition, genistein at a higher dose (5 μg/g) augmented uterus weight in OVX rats (128.6±6.2 mg) compared to OVX controls (96.1±7.5 mg, P<0.05).

3.2. OT and OTR expression

To define cell type, OT and OTR localization was mRNA (2-fold, P<0.05), with a slight increment of ERα expression in 2-fold, P<0.05). These results show that genistein increased OT, OTR- and eNOS-specific mRNA (3- to 4-fold, P<0.05), but induced a 50% fall in ERβ mRNA (2-fold, P<0.05). These results were confirmed by similar changes in protein levels, ascertained by Western blotting (Fig. 4B).

To further verify that an estrogen-mediated mechanism induces OT, OTR and eNOS mRNA elevation in the rat aorta, we administered E2 (0.1 μg/g) to OVX rats. As seen in Fig. 5A, increases of aortic OT mRNA (2-fold, P<0.05), OTR mRNA (4-fold, P<0.05) and eNOS mRNA (3-fold, P<0.05) were evident in E2-treated, OVX rats compared to their controls.

As presented in Fig. 5B, E2 treatment also elevated ERα mRNA (2-fold, P<0.05), with a slight increment of ERβ mRNA (1.5-fold, P<0.05). These results show that genistein and E2 regulate aortic ERβ mRNA expression in different ways.

Table 2
Effect of genistein treatment on body weight, uterine weight, blood pressure (BP), heart rate (HR) and serum concentrations of estradiol and progesterone

<table>
<thead>
<tr>
<th></th>
<th>Sham+ vehicle (n=10)</th>
<th>OVX+ vehicle (n=10)</th>
<th>OVX+ genistein (1 μg/g bw, n=10)</th>
<th>OVX+ genistein (5 μg/g bw, n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔBody weight (g)</td>
<td>31.8±3.6</td>
<td>43.1±2.5</td>
<td>34.1±2.3</td>
<td>29.9±1.7*</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>474±66</td>
<td>96±7*</td>
<td>113±6*</td>
<td>128±6#</td>
</tr>
<tr>
<td>BP (systolic mmHg)</td>
<td>11±2</td>
<td>120±7</td>
<td>122±5</td>
<td>118±5</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>378±7</td>
<td>403±14</td>
<td>390±10</td>
<td>416±24</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>146±29</td>
<td>&lt;3.7*</td>
<td>&lt;3.7*</td>
<td>&lt;3.7*</td>
</tr>
<tr>
<td>Progesterone (nmol/l)</td>
<td>98.8±18.4</td>
<td>11.2±2.6*</td>
<td>17.1±7.0*</td>
<td>12.2±4.3*</td>
</tr>
</tbody>
</table>

* P<0.05 vs. SHAM; § P<0.05 vs. OVX rats treated with vehicle. bw=body weight.
4. Discussion

This work is a follow-up of experiments on OT function in the cardiovascular system [4–6,10]. In the present paper, we found that OT and OTR are co-localized in aortic endothelial cells of the tunica intima. Furthermore, we observed that genistein and E₂, administered to OVX rats, enhance OT mRNA and OTR mRNA in the rat aorta. eNOS mRNA activation paralleled the increases in OT and OTR mRNA. Western blot analysis showed elevations of 67-kDa OTR and 140-kDa eNOS proteins. Enhancement of aortic OT and OTR by genistein is associated with ERα upregulation and is inhibited by ER blockade with ICI 182,780. Previous studies from this laboratory have demonstrated that OT, synthesized locally and stored in the rat aorta and vena cava, as well as immature female rats respond to diethylstilbestrol treatment with heightened vascular OT and OTR mRNA levels [5]. Correspondingly, the present findings reveal that the OT system in OVX rats is activated by genistein and E₂. In addition, we document that both OT and OTR are abundant in aortic endothelial cells of the tunica intima. This supports the concept that the OT system in the aorta acts locally via an autocrine/paracrine mechanism in the vascular endothelium [1,9].

Our study shows that genistein 1 µg/g (but not the lower doses of 0.02 and 0.1 µg/g) is required to stimulate the OT system in the OVX rat aorta. The 1-µg/g dose induced OT, OTR and eNOS responses in the aorta without an increase in uterus weight, which was found when genistein 5 µg/g was administered to OVX rats. This implies that dose of genistein is critical in the induction of tissue-dependent biological actions. Similarly, dietary isoflavones at a dose level of 157 mg/kg daily for 1 month (but not 67 mg/kg per day) completely restored the impairment of acetylcholine-induced vasodilation in OVX rats consuming a soy diet, and the effect was associated with uterus enlargement [16–20]. The dietary and pharmacological actions of genistein have already been compared in other studies. For example, in rats, the 16.6-µg/g dose injected and the 250-mg dietary dose per rat result in similar total serum genistein concentrations: 1380 and 1115 nM, respectively [22]. However, all these studies reported the physiologic but not the genomic effects of genistein treatment. At present, we demonstrate that genistein, similarly to E₂, causes changes in the gene expression pattern of the rat aorta, targeting the intrinsic OT system.

We found that OT and OTR mRNAs in the rat aorta are increased either by genistein or E₂ in parallel to eNOS
mRNA augmentation. With respect to the clinical relevance of this observation, the chronic administration of physiologically relevant doses of OT to rats lowered BP [23], and OT exerted a peripheral vasodilating effect in humans [24]. Mean arterial pressure is also reduced in OVX, spontaneously hypertensive rats consuming a soy diet compared to casein-fed controls [25]. OTR has been recently found in endothelial cells of the human umbilical vein, aorta, and pulmonary artery [9]. In these endothelial cells, OTR activation leads to an increase of intracellular calcium and NO with stimulation of cell growth. Recently, we discovered that in the presence of OT, P-19 stem cells differentiate into cardiac myocyte-like cells [26]. Thus, OTR activation by genistein may induce proliferation of endothelial cells and recovery of impaired endothelial function.

On the other hand, evidence was provided recently for OT-dependent regulation of acetylcholine release from intrinsic cardiac cholinergic neurons [10]. Therefore, upon activation by genistein, the OT system can elicit parasympathetic stimulation via acetylcholine release that would act on muscarinic cholinergic receptors to increase intracellular Ca$^{2+}$ concentrations within vascular cells.
Genistein enhances the expression of the OT system and isoflavones inhibit ERα this brain region [32]. These results, which suggest that isoflavones inhibit ERα in the brain, are in contrast with the finding that genistein acts as an estrogen agonist in the heart [33] and with our observation in the rat aorta. In addition, recent studies in female Cynomolgus monkeys indicate that soy consumption enhances the cardioprotective effects of E2 in regard to coronary artery dilation [34]. These investigations suggest that the mode of genistein action may be tissue-specific.

Furthermore, it has been demonstrated that acetylcholine-induced vasodilation decreased after ovariectomy, and that the dietary isoflavones genistein and daidzein reversed this impairment [12,19,27]. The vasodilatory effect is attributed to genistein, since treatment with the compound (0.2 μg/g for 4 weeks) in similar studies improved the acetylcholine-induced relaxation of aortic rings [12].

Genistein enhances the expression of the OT system and eNOS interacting with ERs. The impact of genistein on uterine ERαs and its uterotrophic effects [27] indicate estrogen agonism. Similar actions can be induced in the aorta of OVX rats because OT and OTR elevation is inhibited by an ER antagonist. Structurally similar to E2, genistein is capable of eliciting numerous biological responses that mimic those of E2 via co-activation of ER. Genistein binds to ERα [28] and has a higher affinity for ERβ [29] that is highly localized in the vascular tree [30]. Our studies demonstrate that E2 and genistein act in different ways on ERα and ERβ in the rat aorta. Genistein decreases ERβ, but enhances ERα, whereas E2 increases ERα and slightly augments ERβ expression. Thus, OTR upregulation in the rat aorta may be mediated by ERα. This is consistent with reports in knock-out mice that ERα is not necessary for basal OTR synthesis but is essential for the induction of OTR binding in the brain by estrogen [31]. Our finding that OTR activation by genistein parallels ERβ reduction is consistent with the work of Patisaul et al. [32] who noted increased OT binding in the ventromedial nucleus of the hypothalamus with ERβ mRNA decreased correspondingly by E2 treatment, but not by dietary phytoestrogens. When applied together, however, phytoestrogens inhibited E2 effects and induced ERβ mRNA in this brain region [32]. These results, which suggest that isoflavones inhibit ERα in the brain, are in contrast with the finding that genistein acts as an estrogen agonist in the heart [33] and with our observation in the rat aorta. In addition, recent studies in female Cynomolgus monkeys indicate that soy consumption enhances the cardioprotective effects of E2 in regard to coronary artery dilation [34]. These investigations suggest that the mode of genistein action may be tissue-specific.

The in vitro experiments of Miller et al. [35] indicate that OT does not have a direct effect on vascular tone in resistance-sized vessels from nonpregnant or pregnant rats. If OT is an important mediator of vascular control, these data suggest that its actions are indirect. It may be argued that genistein does not function here as an estrogen but exerts its effect via other mechanisms, such as activation of calcium channels [36], inhibition of tyrosine kinases [37], or direct interaction with various cellular enzymes [38]. Hence, it is conceivable that the effects of genistein are mediated via some alternative pathways that do not involve ERs. In numerous in vitro studies on vascular SMC, genistein blocked several tyrosine kinase-dependent events that are critical in the regulation of SMC proliferation and contractility [39]. Similarly to the effects of E2, the vasodilatory responses to genistein are only partially endothelium-dependent [39], and there is direct evidence that, through tyrosine kinase inhibition, genistein reduces fura-2-measured intracellular calcium activity in mesenteric arteries stimulated with noradrenaline [40]. However, since the structurally similar but tyrosine kinase-inactive compound daidzein also exhibits vasodilatory capacity, it is clear that some of these effects are unrelated to tyrosine kinase inhibition [41].

In summary, this study shows that the OT system is expressed in endothelial cells of the aortic tunica intima, and genistein treatment stimulates OT and OTR in the
aorta of OVX rats. This action may play a role in the recovery of endothelial function impaired by ovariectomy.

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