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Endogenous oestrogens do not regulate endothelial nitric oxide production in early postnatal rats

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A B S T R A C T

Previously we showed that endothelium of 1-2-weeks old rats exerts an anticontractile effect due to spontaneous NO production which correlates with a higher eNOS expression level compared to adult rats. Oestrogens are powerful regulators of eNOS expression and activity in arterial endothelium. This study tested the hypothesis that anticontractile influence of endothelium in young rats is regulated by endogenous oestrogens. Wistar rats were daily treated with ICI 182,780 or letrozole (oestrogen receptor antagonist and aromatase inhibitor, respectively; s.c., 1 mg/kg/day) from the second postnatal day, control pups received vehicle injections. At the age of 10–12-days we studied contraction of saphenous arteries using wire myography. ELISA and qPCR were used to evaluate blood sex steroids levels and mRNA expression in arterial tissue, respectively. Ten-12 days old male rats compared to adult male rats demonstrated 78% higher serum 17β-oestradiol concentration and several-fold increase in mRNA contents of oestrogen receptors (ERα and GPER1). However, treatments with ICI 182,780 or letrozole did not affect arterial sensitivity to methoxamine (α_1-adrenoceptor agonist) in 10–12-days old males. The blockade of NO-synthase with L-NNA caused tonic contraction and potentiated the response to methoxamine, these effects were similar in control and both treated groups. The sensitivity of endothelium-denuded saphenous arteries to NO-donor DEA/NO did not differ between control and treated groups as well. In addition, treatments with ICI 182,780 or letrozole did not change eNOS expression level in arterial tissue. Our results suggest that endogenous oestrogens do not regulate anticontractile effect of NO during early postnatal development in rats.

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1. Introduction

The processes of growth and development of the organism are associated with numerous structural and functional changes in circulatory system including vascular endothelium (Boegehold, 2010). In healthy organism endothelium produces factors, which weaken vasoconstrictor influences (so-called anticontractile effect of endothelium). Nitric oxide (NO) is a key relaxing factor produced by vascular endothelium. Previously we showed that endothelium of 1–2 weeks old rat pups in contrast to adult rats tonically produces NO (Gaynullina et al., 2013). Such an influence of endothelium in young rats is associated with increased expression level and activity of endothelial NO-synthase (eNOS) (Gaynullina et al., 2013). However it remains unclear which regulatory factors are responsible for the formation of such neonatal phenotype of endothelial secretion.

Oestrogens are known to be powerful regulators of eNOS (Duckles and Miller, 2010). They exert a long-term genomic influence on NO production in the endothelium by increasing the expression levels of eNOS mRNA and protein (Kleinert et al., 1998; MacRitchie et al., 1997). In addition, rapid action of oestrogens through membrane receptors leads to PI3-kinase/Akt pathway activation and eNOS phosphorylation at the Ser1177 site (Duckles and Miller, 2010; Kim and Bender, 2009). Oestrogenic effects on eNOS are mainly mediated through oestrogen receptor α (ERα), which is localised in nucleus and cell membrane (Kim et al., 2014) and also through G-protein-coupled oestrogen receptor (GPER1) (Lindsey et al., 2014).

The level of oestrogens is known to be much higher in blood of rats during first days after birth compared to adult animals (Cheng

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and Johnson, 1973; Ojeda et al., 1975; Weisz and Gunsalus, 1973). The main source of oestrogens in early postnatal period are adrenal glands (Weisz and Gunsalus, 1973), that produce dehydroepiandrosterone (DHEA), metabolic precursor of androgens and oestrogens (Luu-The and Labrie, 2010). Another important source of oestrogens is their local conversion from androgens by aromatase, which plays a special role in NO-dependent endothelial function in males (Gonzales et al., 2007; Kimura et al., 2003). Nevertheless, the role of oestrogens in regulation of endothelial NO production in early postnatal period is poorly understood.

The aim of this study was to test the hypothesis that the increased expression level and activity of eNOS in early postnatal ontogenesis in rats are under the control of oestrogens. First, we compared sex steroid levels in blood and oestrogen receptors expression levels in arterial tissue of young and adult rats. Second, we studied endothelial function in young male rats chronically treated with ERα/β blocker or aromatase inhibitor.

2. Materials and methods

2.1. Animals

All experiments and procedures in this study were performed in full compliance with the NIH Guide for the Care and Use of Laboratory Animals and Russian national guidelines for animal research. Experiments were held on young (10–12 days old) and adult (2–3 months old) male Wistar rats.

2.2. Measurement of blood parameters

After decapitation blood samples were collected and then incubated for 30 min at 37 °C and 30 min at 4 °C. Then the samples were centrifuged (4300 g, 15 min) and the serum was collected and kept frozen at −20 °C till the analysis.

Measurement of sex steroids levels in blood serum was performed by ELISA. We used commercial kits from Immunotek (Moscow, Russia) for 17β-oestradiol and testosterone and a commercial kit from Diagnostics Biochem Canada (Dorchester, Ontario, Canada) for DHEA-sulphate (DHEAS).

For measurement of NO metabolites in blood serum we used Griess reaction (Giustarini et al., 2008). Briefly, equal volumes of serum and $ZnCl_2$ (0.5 M) were mixed and centrifuged (15 min at 6700 g) to deproteinate the samples. For lipid extraction an equal volume of chloroform was added to the supernatant, the probes were mixed and centrifuged (10 min at 6700 g). Then samples were incubated with equal volume of 0.8% VCl_3 for the reduction of nitrates to nitrites simultaneously with Griess reaction (Miranda et al., 2001). Optical density was measured in a spectrophotometer (Jenway 6715) at 543 nm. The level of NO metabolites in serum was calculated respective to calibration curve for sodium nitrite (from 0.5 μmol/l to 30 μmol/l).

2.3. Measurement of mRNA level in arterial preparations by qPCR

In arterial preparations from adult and young rats the following genes expression levels were evaluated: eNOS, ERα, GPER1, aromatase and three house-keeping genes (GAPDH, 18S and $RPLP_0$). All primers were obtained from Evrogen (Moscow, Russia), their sequences are listed in Table 1.

For preparation of one tissue sample one saphenous artery from adult rat or two saphenous arteries from young rat were isolated and stored at −80 °C in RNA-later (Qiagen). For RNA extraction we used ExtractRNA solution from Evrogen (Moscow, Russia). RNA concentration was measured in Nanodrop 1000 (Thermo Scientific, USA) and then all samples were diluted to concentration of 10 ng/μl. After that the samples were treated with DNase I (Fermentas, 1000 U/ml) and cDNA was synthesized using reverse transcription kit MMLV RT kit (Evrogen). qPCR was run in Rotor Gene 6000 (Corbett Research, Australia) using SYBR Green I and Taq-polymerase Master Mix (Syntol, Moscow, Russia). Results were analyzed in Rotor Gene 6000 Software and gene expression levels were calculated as $1/E^{C_T}$, where E – primer efficiency, C_T – cycle number on which the curve for product accumulation is crossing the fluorescence detection threshold. These values were normalised to the geometric mean of the three housekeeping genes in the same sample.

Table 1
Gene specific primers used in qPCR.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
eNOS	GGATTCTGGCAAGACCGATTAC	GGTGAGGACTTGTCCAAACACT
ERα	GGAGACTCGCTACTGTGCTGTGT	AGTCATCTCTCTGACGCTTGTGC
GPER1	CGCTCAAGGCAGTCATACCA	CCCCTGTCCGTTTTCCTCTA
Aromatase	CCTGGAGGATGACGTGATTG	CGATGTACTTCCCAGCACAG
GAPDH	CACCAGCATCACCCCATTT	CCATCAAGGACCCCTTCATT
18S	CACGGGTGACGGGGAATCAG	CGGGTCGGGAGTGGGTAATTTG
$RPLP_0$	AGGGTCCTGGCTTTGTCTGTGG	AGCTGCAGGAGCAGCAGTGG

2.4. Chronic treatment with ERα blocker or aromatase inhibitor in early postnatal period

We used ICI 182,780 (Fulvestrant, Sigma-Aldrich) as a selective antagonist of ERα/β and letrozole (Sigma-Aldrich) as nonsteroid competitive inhibitor of aromatase. Male rat pups were treated with ICI 182,780 or letrozole subcutaneously in a dosage of 1 mg/kg/day from the second postnatal day till the day preceding the experiment. Control pups were injected with the same volume (2.5 μl per gram of body weight) of vehicle (peach oil). Pups were taken into *in vitro* experiments at the age of 10–12 days.

2.5. Experiments on isolated arteries

To record isometric force 2-mm long segments were isolated from saphenous artery of young rats and mounted in a wire myograph (410A or 620M, DMT A/S, Denmark). Transducer readings were digitalised at 10 Hz and recorded on PC hard drive using analogue–digital converter (E14-140M, L-CARD, Russia) and PowerGraph 3.3 software (DISoft, Russia). The solution in myograph chamber contained (in mM): NaCl 120; $NaHCO_3$ 26; KCl 4.5; $CaCl_2$ 1.6; $MgSO_4$ 1.0; NaH_2PO_4 1.2; D-glucose 5.5; EDTA 0.025; HEPES 5.0. Throughout the experiment the solution was kept at 37 °C and bubbled with gas mixture (95% O_2, 5% CO_2) to set pH at 7.4. Firstly we constructed the passive elastic characteristic of the preparation to determine the inner wall circumference at which the segment develops maximal active tension (Mulvany and Halpern, 1977). Then arterial segments were activated once with norepinephrine (10 μM, Sigma-Aldrich) and twice with methoxamine (α_1-adrenoreceptor agonist, 10 μM, Sigma-Aldrich). To evaluate endothelial function we added acetylcholine (10 μM, Sigma-Aldrich) on top of the second methoxamine-induced contraction. Endothelium was considered intact when relaxation was more than 70% from the level of pre-constriction.

Twenty-five min after the activation procedure we started cumulative addition of methoxamine (in the range from 0.01 μM to 100 μM) (Fig. 1). Following first concentration–response relationship and 15-min washout one arterial segment was incubated with NO-synthase inhibitor N–nitro-L-arginine (L-NNA, Alexis Biochemicals, 100 μM) (Fig. 1A) and another with its inactive analogue N–nitro-D-arginine (D-NNA, Alexis Biochemicals, 100 μM) (Fig. 1B). After the incubation period (25 min) the second

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