

Online Data supplement

Estradiol Promotes Sudden Cardiac Death in Transgenic Long-QT Type 2 Rabbits while Progesterone is Protective

Methods

All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guidelines after approval by the Institutional Animal Care and Use Committee.

Ovariectomy, hormone pellet and telemetric ECG device implantation

Ovariectomy and sham surgeries were performed in prepubertal LQT2 rabbits that were anesthetized with ketamine, xylazine, and buprenorphine (25 mg/kg; 3.75 mg/kg; 0.03 mg/kg IM), intubated and ventilated with isoflurane (0.5-2% in 0.5 O₂). 90-day release pellets (Innovative Research of America) containing 17 β -estradiol (150 mg), dihydrotestosterone (200 mg), progesterone (150 mg), or placebo were implanted subcutaneously. Telemetric ECG devices (F70-EEE, Data Sciences International) were implanted subcutaneously on the rabbits' back, and pairs of electrodes were tunnelled to the right shoulder, left lateral thorax, and both hips as described⁸.

Blood samples were obtained at baseline, after 30 and 60 days of hormone-treatment. Serum hormones were extracted using diethyl-ether, and the levels were quantified using ELISA kits (11-DHTHU-E01, 11-PROHU-E01, 20-DR-4399, ALPCO Diagnostics). As described previously^{12, 13}, treatment with 90-day release pellets resulted in significantly (10-15 times) higher hormone levels than in sham-operated or ovariectomized females (Fig. 1 supplement), reaching estradiol levels similar to women during the follicular phase, progesterone levels comparable to pregnant rabbits (Lau et al., 1984), and dihydrotestosterone levels similar to sham-operated males. Transthoracic echocardiography was performed after 8 weeks hormone-treatment (parasternal long axis, 7.5 MHz probe) to exclude pro-hypertrophic hormone effects (Table 1 supplement).

Telemetric ECG monitoring: QT/RR ratio and arrhythmia screening

Analog telemetric ECG signals were acquired by Dataquest ART data acquisition software (DSI) and were analyzed using Ponemah ECG analysis software (DSI)^{8, 9}. Pairs of QT/RR intervals were averaged over 5 s every 20 min during a 24-hour monitoring period. QT/RR ratio was calculated using linear regressions and QT/RR ratio at baseline and after 4 weeks of hormone-treatment were compared. To evaluate sex hormone effects on QT duration irrespective of the heart rate, we applied the following previously established heart rate correction formula⁸: LQT2: QT expected = 35 + 0.50 * RR. The QT-index (QT_i) was defined as the percentage of the observed vs. the expected QT

Rabbits were screened for arrhythmias (atrio-ventricular (AV) blocks, premature ventricular contractions (PVCs), bigeminy, couplets, triplets, non-sustained ventricular tachycardia (VT) < 30 s duration, and sustained VT > 30 s duration) within corresponding 2-hour intervals one week prior to electrophysiological studies (EPS) and within 96 hours post EPS and arrhythmias were classified using Lown's classification¹⁴. In rabbits that survived, arrhythmias were analyzed in two 2-hour intervals – 2-4 hours and 10-12 hours after EPS – which were defined by the different time points of death in four rabbits. In rabbits that died within the 96 hours period, arrhythmias were analyzed at the 2-4 hours interval as well as during the 2 hours prior to death. Similarly as in clinical LQTS studies³, we assessed the incidence of the compound endpoint major life-threatening cardiac events – defined as pVT and SCD – during the 8 weeks of hormone-treatment.

***In vivo* electrophysiological studies (EPS)**

After 8 weeks of hormone-treatment, catheter-based *in vivo* EPS were performed¹⁵ in *n*=6 rabbits per group. Under isoflurane anesthesia (0.5-2% in 0.5 O₂) steerable quadripolar and decapolar 4F EP-catheters (Irvine Biomedical) were placed in the right atrium and right ventricle via the right femoral and jugular veins. ECG signals were recorded continuously using the EP-Bard-System Software OS2/warp (kindly provided by Bard Electrophysiology),

filtered with a bandwidth of 30–250 Hz (intracardiac signals) or 0.01–100 Hz (surface ECG). EPS were performed at a stimulation cycle length (CL) of 300 and 240 ms at twice the threshold. Ventricular effective refractory periods (VERP) in RVapex and septal-base position were assessed by progressively shortening the S2-interval in 10-ms steps after 8-beat S1 trains. VERP-dispersion, defined as difference between VERP_{base} and apex, was calculated. Complete EPS were performed at baseline and during isoproterenol infusion (0.10 - 0.25 µg/min to increase the spontaneous heart rate to 120%).

Optical mapping

Heart preparation and retrograde perfusion were performed as described⁸ in $n=4-7$ rabbits per group one week after EPS. Blebbistatin (5 µM) was added to the perfusate to reduce motion artefacts (Fedorov et al., 2007). We used a dual voltage-calcium camera system (100x100 pixels, Ultima-L, Scimedica, Japan)¹⁶ and fluorescence probes PGH1 for membrane potential (V_m) (generously provided by Dr. Guy Salama, University of Pittsburgh) and rhod-2 for Ca_i (Invitrogen). Images were acquired from the anterior surface of the LV and the field of view was set to $1.5 \times 1.5 \text{ cm}^2$ with a spatial resolution of $150 \times 150 \text{ µm}^2$. Hearts were stimulated with progressively faster pacing until either loss of 1:1 capture or VF induction occurred. Sampling rate was set to 1000 f/s, and data were analyzed with custom-built software using Interactive Data Language (ITT Visual Information Solutions) as described in detail^{8, 16}. Action potential duration (APD) maps were calculated by averaging 5 consecutive beats at 350 ms stimulation CL. To investigate hormone effects on early afterdepolarisation (EAD) formation, hearts were exposed to an intra-coronary isoproterenol bolus (140 nM) after performing AV-node ablation with an electrocautery to slow the heart rate (similar to observed AV block prior to *in vivo* pVT).

Patch clamp

Isolation of cardiomyocytes by standard enzymatic techniques and patch-clamp recordings were performed as described⁸. Apical ventricular myocytes were isolated from

hearts of $n=3$ rabbits per group. Whole-cell recordings (11-18 cardiomyocytes per group) were obtained with an Axopatch-200B amplifier (Axon Instruments) with standard patch-clamp techniques. I_{to} , I_{K1} and $I_{Ca,L}$ were recorded in standard Tyrode solution, while I_{Kr} and I_{Ks} were recorded in low K^+ and low divalent cation solution as described⁸. For I_{Kr} and I_{Ks} recording, holding potential was at -40 mV, test potentials were from -30 to $+30$ mV with steps of 10 mV lasting 3 seconds. Tail current was recorded after the test potential was back to -40 mV. For I_{to} recording, holding potential was at -80 mV and test potentials were from -20 to $+50$ mV with steps of 10 mV lasting 400 ms. For I_{K1} recording, a 2 -second ramp from -120 mV to $+60$ mV was used as stimulation voltage. For $I_{Ca,L}$ recording, holding potential was -50 mV, test potentials were -40 to $+40$ mV with 10 mV steps lasting 250 ms. $I_{Ca,L}$ was defined as the difference of peak and steady state current at the end of pulse. E-4031 (5 μ M) and chromanol 293B (30 μ M) were used for isolating I_{Kr} and I_{Ks} , respectively. Tetrodotoxin (20 μ M) and $CdCl_2$ (0.2 mM) were added to block Na^+ and Ca^{2+} currents.

Western Blot

After 2 months of hormone-treatment, hearts were removed, sectioned, and rapidly frozen in liquid nitrogen. Crude membrane preparations of the LV apex were obtained as described⁸. Membrane protein samples were mixed with 4x Laemli-SDS, heated for 5 min at $95^\circ C$, then loaded onto an 8% acrylamide/SDS gel and run at 55 mA for at least 3 h. Proteins were transferred onto a nitrocellulose membrane (Perkin Elmer) overnight at 54 V at $4^\circ C$ and membranes were blocked with 5% milk/TBS. Anti-SERCA2a (Thermo Scientific, MA3-919), anti-PLN (Thermo Scientific, MA3-922), and anti-NCX (Thermo Scientific, MA3-926) mouse monoclonal were used as primary antibodies and HRP conjugated goat-anti-mouse (IgG polyclonal, Thermo Scientific) as secondary antibodies. GAPDH was used as loading control (Sigma, 68795). For quantification, Image-J was used with the GAPDH signal utilized to correct for loading errors by calculating the ratio of target protein intensity to GAPDH intensity.

Statistical analysis

For normally distributed values, we used Student's *t*-test (paired and unpaired). Chi-square test was used for categorical variables. Analysis was performed with Prism 4.03 for Windows (Graphpad). All data are presented as means \pm SD if not mentioned otherwise, and a *p* value ≤ 0.05 was considered significant.

Figure 1 supplement

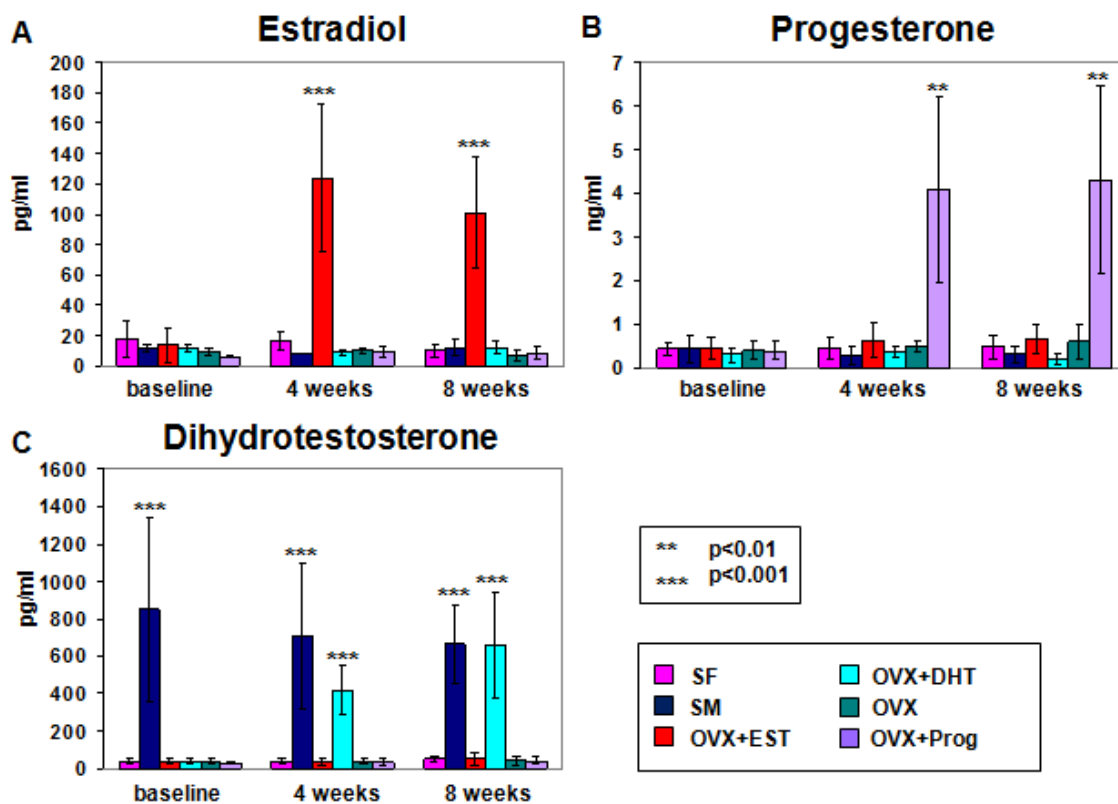


Figure 1 Supplement

Hormone Serum Levels

A.–C. Serum estradiol (A, EST, pg/ml), progesterone (B, PROG, ng/ml), and dihydrotestosterone (C, DHT, pg/ml) levels in sham-operated females (SF), and males (SM), EST, DHT, OVX, and PROG-treated females (*n*=12) at baseline (before ovariectomy surgery), and after 4 and 8 weeks of hormone treatment. ** *p*<0.01; *** *p*<0.001. All values are shown as mean \pm SD.

Table 1 Supplement

Echocardiographic parameters

	IVSd (mm)	LVPWd (mm)	LVIDd (mm)
SF	2.73±0.01	2.66±0.11	10.5±1.05
SM	2.87±0.03	2.95±0.12	10.3±1.15
EST	2.67±0.27	2.73±0.23	11.7±0.42
DHT	2.84±0.07	2.80±0.23	11.7±0.14
OVX	2.62±0.14	2.80±0.28	9.97±0.73
PROG	2.79±0.26	2.87±0.09	9.82±0.1

Table 1 Supplement: Echocardiographic parameters (IVSd, diastolic wall thickness of interventricular septum; LVPWd, diastolic wall thickness of LV posterior wall; LVIDd, diastolic internal LV diameter) in ($n=3$) hormone-treated ovariectomized female (EST, DHT, PROG, and OVX), sham-operated female (SF), and male (SM) LQT2 rabbits.

Additional references

Lau IF, Saksena SK, Salmons R: The concentrations of progesterone, 20 alpha-dihydroprogesterone, testosterone, oestrone and oestradiol-17 beta in serum, amniotic fluid and placental tissue of pregnant rabbits. *Acta Endocrinol* 1982; 99:605-611.

Fedorov VV, Lozinsky IT, Sosunov EA, et al.: Application of blebbistatin as an excitation-contraction uncoupler for electrophysiologic study of rat and rabbit hearts. *Heart Rhythm* 2007; 4:619-626.