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Crucial Role of Phospholamban Phosphorylation and S-Nitrosylation in the Negative Lusitropism Induced by 17β-estradiol in the Male Rat Heart

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Key Words
17beta-estradiol • Lusitropy • Rat Langendorff heart

Abstract
Background/Aims: 17β-estradiol (17βE2) plays an important cardiovascular role by activating estrogen receptors (ER) α and ERβ. Previous studies demonstrated that the novel estrogen G protein-coupled receptor (GPR30/GPER) mediates estrogen action in different tissues. We have recently shown in the rat heart that 17βE2 elicits negative inotropism through ERα, ERβ and GPR30, by triggering activation of ERK1/2, phosphatidylinositol 3-kinase (PI3K), protein kinase A (PKA) and endothelial Nitric Oxide synthase (eNOS) signaling. Methods: In the present study, using the isolated and Langendorff-perfused rat heart as a model system we analyzed: i) whether and to which extent 17βE2 modifies mammalian ventricular myocardial relaxation (lusitropism); ii) the type of ERs and the signaling pathways involved in this effect. Results: We found that 17βE2 negatively modulated the ventricular lusitropic performance. This effect, which partially involved the vascular endothelium, recruited ERβ and occurred via PI3K, eNOS-NO-cGMP-protein kinase G (PKG) transduction cascade. Of note, 17βE2-mediated negative lusitropism associated with a modification of phospholamban (PLN) phosphorylation and S-nitrosylation (SNO) both in isolated Langendorff rat heart and in isolated cardiomyocytes. Conclusion: Taken together, our results allow including 17βE2 to the family of substances that control ventricular relaxation. This is of relevance in relation not only to the normal endocrine control of cardiac function, but also to physio-pathologic conditions characterized by an altered ventricular diastolic performance.

Introduction

17β-Estradiol (17βE2) is a ubiquitous sex steroid hormone with an important cardiovascular activity. Evidences accumulating in males illustrate the multiplicity of estrogen-induced effects on both cardiac and vascular performance. In young men, inhibition of aromatase, the enzyme responsible for a key step in the estrogens biosynthesis, results in impaired flow-mediated vasodilation of the brachial artery [1], while

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absence of functional estrogen receptor α (ERα) induces abnormalities in flow-mediated endothelial vasodilation and premature coronary arterial calcification [2, 3]. These vascular effects are confirmed in male aromatase knockout (ArKO) mouse in which 17βE2 deficiency is associated with impaired endothelium-dependent vasorelaxation [4]. Of note, intracoronary infusion of 17βE2 was found to attenuate ET-1 (endothelin-1)-induced coronary vasoconstriction [3]. This estrogen-dependent cardiovascular protection is confirmed by studies which revealed that men with a genetic variation in ERα have a substantially increased risk for myocardial infarction [5].

17βE2, in addition to its signaling via ligand-regulated transcription factors of the nuclear receptor superfamily, elicits rapid non-genomic responses via membrane-associated receptors which rapidly stimulate multiple intracellular signaling pathways [6]. Recent studies revealed that a novel estrogen receptor named GPR30, belonging to the family of seven-transmembrane G-protein-coupled receptors, significantly contributes to many rapid biological responses elicited by 17βE2 [6]. Therefore, it is proposed that the integration of cell-surface and nuclear signalling impacts overall cell biology. Consistent with this view, we recently demonstrated on the isolated and perfused rat heart that 17βE2 negatively affects cardiac performance by inducing a dose-dependent reduction of contractility via activation of ERα, ERβ and GPR30. This rapid inotropic response recruited ERK (Extracellular signal-regulated kinase), PI3K (Phosphatidylinositol 3-kinase), PKA (protein kinase A), and eNOS (endothelial Nitric Oxide Synthase) [7].

In recent years, great attention has been focused on cardiac relaxation (lusitropism), being this a crucial component of the cardiac cycle. In fact, a proper relaxation (i.e. restoring the diastolic ventricular pressure after each contraction) allows the ventricle to be adequately filled with blood, with a notable influence on the subsequent contraction. Additionally, an impaired relaxation strongly contributes to cardiac dysfunction in important cardiovascular diseases such as heart failure with normal ejection fraction [8]. Many endogenous and exogenous substances are able to interfere with relaxation either decelerating (negative lusitropic) or hastening (positive lusitropic) it both under physiological conditions and in the presence of pathological challenges. An example is provided by β_3 adrenergic receptor activation [9], and a number of Chromogranin A (CGA)-derived peptides [10-12] which induce negative lusitropic responses.

The present study was designed to evaluate in the isolated and Langendorff perfused male rat heart whether, in addition to its negative inotropic action [7], 17βE2 is able to influence ventricular relaxation. Attention has been paid to the transduction pathways recruited by 17βE2 to influence lusitropism, with focus on the specific lusitropic modulator phospholamban (PLN). Since S-nitrosylation has emerged as an important mechanism that, together with phosphorylation, dynamically regulates many proteins [13] including PLN [14], the effect of 17βE2 will be analysed in terms of phosphorylation/nitrosylation of PLN.

Materials and Methods

Animals

Adult Wistar rats (250–300 g body weight; Harlan, Italy), fed a standard diet and water *ad libitum*, were used. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (publication No. 85–23, revised 1996).

Perfusion technique

Rats were anesthetized by ethyl carbamate (2 g/kg body weight, i.p.). Hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated via the aorta and perfused in the Langendorff mode at a constant flow-rate of 12 ml/min and temperature of 37°C. The perfusion solution was a modified Krebs-Henseleit buffer gassed with 95 % O_2 and 5 % CO_2 (pH 7.4) containing (in mmol/l): NaCl 113.0; KCl 4.7; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; $CaCl_2$ 1.8; glucose 11, mannitol 1.1, Na-pyruvate 5 [12].

Left ventricular pressure was measured by means of a latex water-filled balloon inserted into the left ventricle via the left atrium [adjusted to obtain left ventricular end-diastolic pressure (LVEDP) of 5–7 mmHg] and connected to a pressure transducer (BLPR gauge, WRI, Inc. USA). The maximal values of the first derivative of LVP, [+(LVdP/dt)max, mmHg s^{-1}], which indicates the maximal rate of left ventricular contraction, the time to peak tension of isometric twitch (TTP, s), the maximal rate of left ventricular pressure decline of LVP [-(LVdP/dt)max, mmHg s^{-1})], the half time relaxation (HTR, s), which is the time required for intraventricular pressure to fall from the peak to 50% [15], Tau (τ, s), which is the relaxation time constant; the T/-t ratio obtained by +(LVdP/dt)max/-(LVdP/dt)max [16], as indexes of contraction and relaxation, and the left ventricular end-diastolic pressure (LVEDP) were used to assess cardiac function. Mean Coronary Pressure (CP, mmHg) was calculated as the average of values obtained during several cardiac cycles.

Ventricular cell isolation

Young adult rats were anesthetized with ethyl carbamate (2 g/kg body weight, i.p.). Hearts were explanted and washed in modified Ca^{2+}-free Tyrode solution contained (mM) NaCl 135, KCl_4, $MgCl_2$ 1, HEPES 2, glucose 10, butanedione monoxime

10, and taurine 5, with pH adjusted to 7.40 with NaOH. Then the hearts were cannulated via the aorta. All operations were carried out under a laminar flow hood. The heart was perfused at a constant flow rate of 10 ml/min with Ca^{2+}-free Tyrode solution with a peristaltic pump for ~5 min at 37°C to wash away the blood and then with 10 ml of Ca^{2+}-free Tyrode solution supplemented with collagenase (0.3 mg/ml) and protease (0.02 mg/ml). Hearts were then perfused and enzymatically dissociated with 30 ml of the Ca^{2+}-free Tyrode solution containing 50 μM $CaCl_2$. Atria and ventricles were separated, and the ventricles were cut into small pieces and shaken for 10 min in 20 ml of Ca^{2+}-free Tyrode solution containing 50 μM $CaCl_2$, collagenase, and protease.

Cardiomyocytes were plated on laminin-treated dishes and incubated in DMEM medium (JRH Biosciences, Sigma-Aldrich Srl., Milan, Italy) supplemented with 5% fetal bovine serum (JRH Bioscience, Sigma-Aldrich Srl., Milan, Italy) and 100 μg/ml penicillin/streptomycin (Sigma-Aldrich Srl., Milan, Italy). Cardiomyocytes were placed in a 37°C, 5% CO_2 incubator until adhesion (2 h).

Western blotting analysis

Cardiac ventricles perfused with either increasing concentrations of 17βE2 (1 pM-10 nM), or PPT (1 pM-10 nM), or DPN (1 pM-10 nM), or with 17βE2 (1 nM) plus H-89 (10 nM) were homogenized in ice-cold RIPA buffer (Sigma-Aldrich, Milan, Italy) containing a mixture of protease inhibitors (1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride and 200 mmol/L sodium orthovanadate). Cardiomyocytes were treated with 17βE2 (10 nM), incubated for 15 min at 37°C and then were lysated in the same lisys buffer. Cardiac tissue homogenate and cardiomyocytes lysates were then centrifuged at 200 g for 10 min at 4°C to remove tissue debris.

10–50 μg of protein of cardiac tissue or cardiomyocytes pellet were electrophoresed through a reducing SDS/15% (w/v) polyacrylamide gel and electroblotted on to a nitrocellulose membrane. After the transfer, the membranes were stained with Red Poinseau to confirm the equal loading and transfer. The membrane was blocked and incubated with the polyclonal IgG for $pPLN^{16}$Ser and $pPLN^{17}$Thr (all purchased from Santacruz, DBA Srl, Milan, Italy). PLN total (pentameric) was used as loading control. The levels of proteins and phosphoproteins were detected with horseradish peroxidase-linked secondary antibodies and the ECL® (Enhanced chemiluminescence) System (GE Healthcare, Milan, Italy). Autoradiographs were scanned to obtain arbitrary densitometric units. Data were normalized against those of the corresponding total PLN. The experiments were performed in triplicate and the results calculated as mean±SD, and expressed as protein change (%).

Nitrosylation

The SNO of proteins was obtained through Biotin switch assay. This protocol was performed as previously described [17]. Extracts were adjusted to 0.5 mg/ml of protein and equal amounts were blocked with 4 volumes of blocking buffer (225 mmol/l Hepes, pH 7.7, 0.9 mmol/l EDTA, 0.09 mmol/l neocuproine, 2.5% SDS, and 20 mmol/l MMTS) at 50 °C for 20 min with agitation. After blocking, extracts were precipitated with 2 volumes of cold (-20 °C) acetone, chilled at -20 °C for 10 min, centrifuged at 2000 g, 4 °C for 5 min, washed with acetone, dried out at room temperature and resuspended in 0.1 ml HENS buffer (250 mmol/l Hepes, pH 7.7, 1 mmol/l EDTA, 0.1 mmol/l neocuproine, and 1% SDS) x mg of protein. Until this step, all operations were carried out in the dark. A 1/3 vol of biotin-HPDP 4 mmol/l in DMF and ascorbate 1 mmol/l were added and incubated for 1 h at room temperature. Proteins were acetone precipitated again and resuspended in the same volume of HENS buffer. To detect biotinylated proteins by Western blot, samples from the biotin switch assay were separated on 15% SDS-PAGE gels, transferred to PVDF membranes, blocked with non fat dried milk, and incubated with streptavidin-peroxidase diluted 1/5000 for 1 h. In additional experiments, the membrane for SNO detection was stripped and reprobed using an anti-PLN antibody (Santa Cruz Biotechnology). Blots were developed by enhanced chemioluminescence (ECL) and were placed in a film cassette with photograph film. Films were exposed for 30 s, developed and fixed.

Experimental protocols

Isolated Langendorff preparation. 17βE2 stimulated preparations. Preliminary experiments (data not shown) obtained by repetitive exposure of each heart to one concentration of 17βE2 (1 nM) revealed absence of desensitization. Thus, concentration-response curves were obtained by perfusing the cardiac preparation with KHs enriched with increasing concentrations of 17βE2 (1 pM-10 nM) for 10 min.

Involvement of estrogen receptors. To demonstrate the involvement of ERs in 17βE2 mediated effects, hearts were perfused with 1 nM 17βE2 for 10 min and washed-out with KHs. Subsequently, cardiac preparations were perfused with ICI (100 nM), a non-selective ER antagonist, for 10 min, followed by perfusion with KHs containing a single concentration of 17βE2 (1 nM) plus ICI (100 nM) for another 10 min. To explore the involvement of ERα, ERβ and GPR30, concentration-response curves were generated using selective agonists. Curves were obtained by perfusing the cardiac preparation with KHs enriched with increasing concentrations (1 pM-10 nM) for 10 min of PPT, a selective ERα agonist [18], DPN, a selective ERβ agonist [19] or G-1, a specific GPR30 agonist [20].

To evaluate whether Gi/o proteins are involved in the cardiac action of 17βE2, hearts were pre-incubated for 60 min with KHs enriched with PTx (0.01 nM) and then exposed for 10 min to 17βE2 (1 nM). As shown in the rat heart [21], PTx catalyses the ADP ribosylation of the a-subunit of Gi/o proteins and uncouples the interaction between Gi and inhibitory receptors of adenylate cyclase.

Based on the results obtained from dose-response curves, antagonists were used at a concentration which did not affect cardiac performance.

Inhibitor stimulated preparations. To verify the pathways involved in estrogen receptor-mediated mechanism, hearts, stabilized for 20 min with KHs, were perfused with 1 nM of 17βE2 for 10min and then washed-out with KHs. After returning to control conditions, each cardiac preparation was perfused

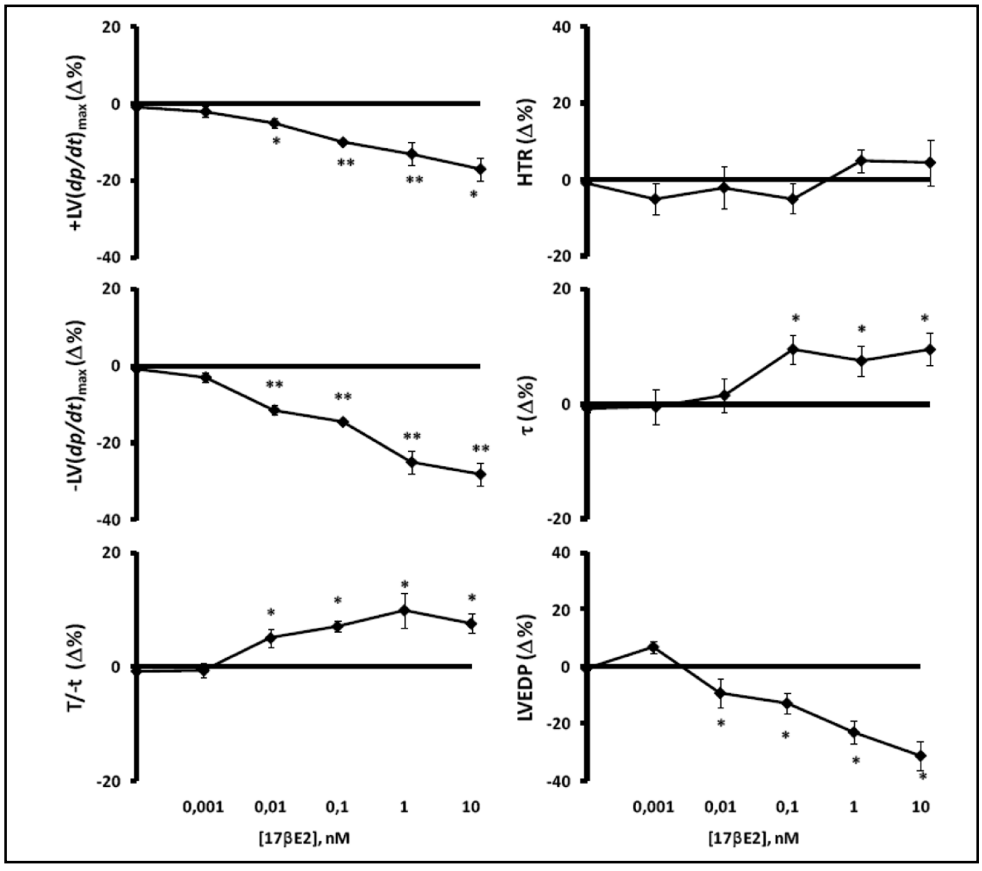

Fig. 1. Concentration-response curves of increasing concentrations (1pM-10nM) of 17βE2 on $+(LVdP/dt)_{max}$, $-(LVdP/dt)_{max}$, T/-t, HTR, τ and LVEDP on the rat isolated and Langendorff perfused rat heart. For abbreviations and basal values, see *Results*. Percentage changes were evaluated as means±SE of 6 experiments for each group. Significance of difference from control values (one-way ANOVA): *P<0.05, **P<0.01.

with a specific inhibitor for 10 min; then it was perfused with KHs containing a single concentration of 17βE2 (1 nM) plus the inhibitor for an additional 10 min. In particular, the involvement of ERK, PI3K and PKA was evaluated by perfusing the hearts with PD (100 nM), LY (100 nM) and H-89 (10 nM), respectively. The involvement of the NO-cGMP pathway was detected by application of L-NIO (selective eNOS inhibitor; 10 μM), ODQ [soluble Guanylate Cyclase (sGC) inhibitor; 100 μM] and KT5823 [Protein Kinase G (PKG) inhibitor; 100 nM].

Endothelium involvement. To evaluate the involvement of the coronary vascular endothelium, coronary artery endothelial dysfunction was achieved by a brief perfusion with Triton X-100 (1:200) administered after 30 min of equilibration. Triton X-100 infusion was equivalent to 1% of the flow rate delivered into the KH buffer immediately above the aorta for 1 s [22]. Subsequently, hearts were perfused for 25 min with normal KHs before the actions of 17βE2 were examined as described above. To confirm the Triton X-100-dependent inactivation of the endothelium, hearts were perfused with bradykinin which was unable to elicit its classic vasodilatory effect (data no shown).

Drugs and chemicals. G-1(1-(4-(6-bromobenzo(1,3)dioxol-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta(c)quinolin-8-yl)ethanone and KT5823 were purchased from Calbiochem (VWR International, Milan, Italy). 17βE2, pertussis toxin (PTx), LY294,002 (LY), PD98059 (PD), H-89, L-N5-(1-iminoethyl)ornithine (L-NIO), 1H-(1,2,4)oxadiazolo-(4,3a)quinoxalin-1-one (ODQ), TritonX-100 were purchased from Sigma-Aldrich. 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), Diarylpropionitrile (DPN) and ICI182,780 (ICI) were obtained from Tocris Chemicals (Bristol, UK). All compounds were solubilized in DMSO, except 17βE2 and PD which were dissolved in ethanol. Preliminary experiments showed that the presence of equivalent amounts of ethanol or DMSO in KHs solution in absence of drugs did not modify basal cardiac performance.

Statistics

Data are the mean±SEM. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the ANOVA test ($p < 0.05$). Comparison between groups was made by using a one-way analysis of variance (ANOVA) followed by the Bonferroni correction for post hoc t-tests. Differences were considered to be statistically significant for $p < 0.05$.

Results

17βE2 effects on lusitropic parameters and receptors characterization

The analysis of the effects of increased concentrations of 17βE2 (1pM - 10nM) revealed that the hormone slowed cardiac relaxation, as shown by the dose-dependent decrease of both $-LV(dP/dt)_{max}$ and $+LV(dP/dt)_{max}$, significant starting from 10 pM (Fig. 1). In addition,

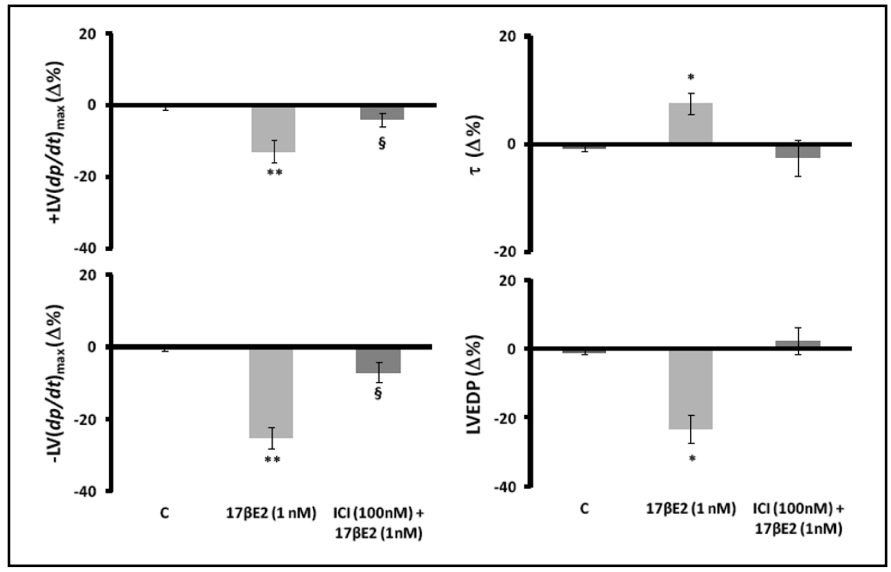

Fig. 2. Effects of 17βE2 before and after treatment with ICI (100 nM) on $+(LVdP/dt)_{max}$, $-(LVdP/dt)_{max}$, τ and LVEDP in the isolated and Langendorff perfused rat heart. Percentage changes were evaluated as means±SE of 6 experiments for each group. Significance of difference (one-way ANOVA) from control values of 17βE2 vs. Krebs-Henseleit solution (KHs): *P<0.05, **P<0.01. Comparison between groups treated with 17βE2 vs. ICI (100 nM): §- p<0.05.

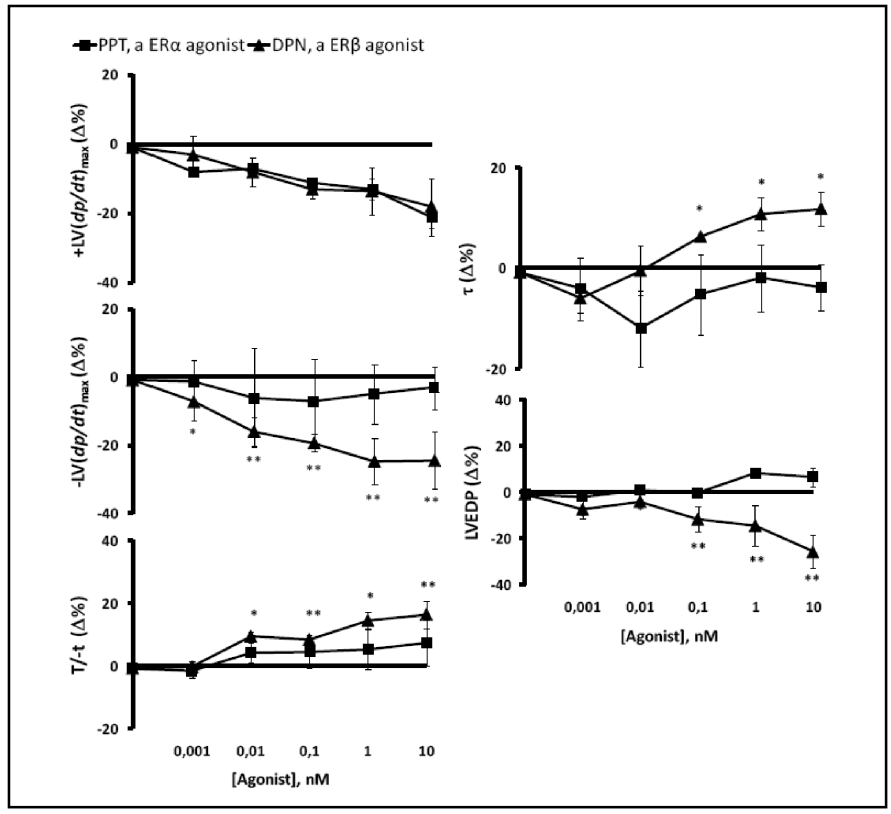

Fig. 3. Concentration-response curves of increasing concentrations (1pM-10nM) of PPT, a selective ERα agonist, and DPN, a selective ERβ agonist, on $+(LVdP/dt)_{max}$, $-(LVdP/dt)_{max}$, T/-t, τ and LVEDP on the rat isolated and Langendorff perfused rat heart. For abbreviations and basal values, see *Results*. Percentage changes were evaluated as means±SE of 5 experiments for each group. Significance of difference from control values (one-way ANOVA): *P<0.05, **P<0.01.

17βE2 induced a dose-dependent increase of T/-t, τ and HTR. The hormone also caused a statistically significant decrease in LVEDP (Fig. 1). [Basal values: $-LV(dP/dt)_{max}$ = -1664 ±70 mmHg s^{-1}; $+LV(dP/dt)_{max}$ = 2489±124 mmHg s^{-1}; T/-t= -1.48±1.85 mmHg s^{-1}; τ= 0.04 ±0.01 s^{-1}; HTR= 0.05 ±0.01 s^{-1}].

The involvement of ERα, ERβ and GPR30 in 17βE2-dependent negative lusitropism was evaluated by perfusing the hearts with the non selective ERs antagonist ICI (100nM). We found that ICI abolished the 17βE2-dependent negative lusitropic effect (Fig. 2). Application of the selective ERs agonists DPN, PPT or G1 (1pM to 10nM) to activate ERα, ERβ and GPR30, respectively, showed that DPN induced a dose-dependent decrease of relaxation. This effect was similar to that elicited by 17βE2. In contrast, both PPT and G1 did not significantly alter cardiac lusitropic parameters, except for the significant decrease in LVEDP which was elicited by both ERβ and GPR30 activation (Fig. 3, 4a) and is accompanied by changes in contraction, as reported in [7].

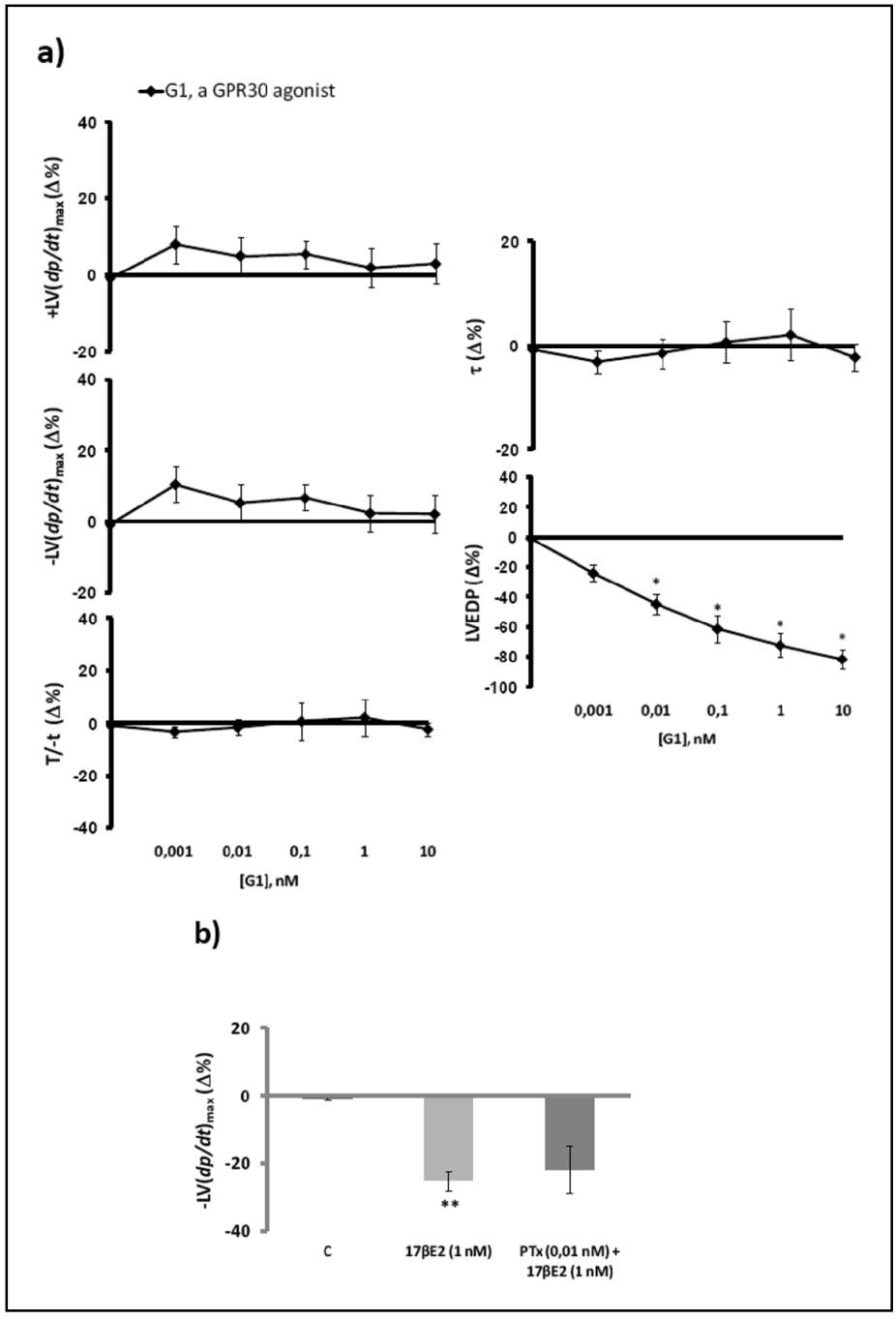

Fig. 4. a) Concentration-response curves of increasing concentrations (1pM-10nM) of G1, a selective GPR30 agonist, on $+(LVdP/dt)_{max}$, $-(LVdP/dt)_{max}$, T/-t, τ and LVEDP on the rat isolated and Langendorff perfused rat heart. b) Effects of 17βE2 before and after treatment with PTx (0,01 nM), on $-(LVdP/dt)_{max}$ on the isolated and Langendorff perfused rat heart preparation. For abbreviations and basal values, see *Results*. Percentage changes were evaluated as means ±SE of 5 experiments for each group. Significance of difference from control values and from control values of 17βE2 alone vs. Krebs- Henseleit solution (KHs) (one-way ANOVA): *P<0.05, **P<0.01. Comparison between groups of 17βE2 alone vs. PTx: §- p<0.05.

To verify the involvement of Gi/o proteins in the mechanism of action activated by 17βE2, cardiac preparations were perfused with KHs containing PTx (0.01 nM) in the presence of the hormone. We found that the toxin didn't affect the 17βE2-dependent negative lusitropism (Fig. 4b), ruling out the involvement of GPR30.

Endothelium involvement

Vascular endothelium is a known estrogen target, because of the presence of all types of ERs [23]. The role of coronary vascular endothelium in the 17βE2-dependent effects on relaxation was examined by inducing endothelial dysfunction through exposure to Triton X100. Results showed that the functional endothelial damage induced by the detergent reduces significantly the negative lusitropic effect elicited by 17βE2 (Fig. 5).

Mechanisms of action of 17βE2

17βE2, via ERβ activation, signals through PI3K, ERK1/2 and PKA [24]. The involvement of this transduction pathway in the 17βE2-dependent negative

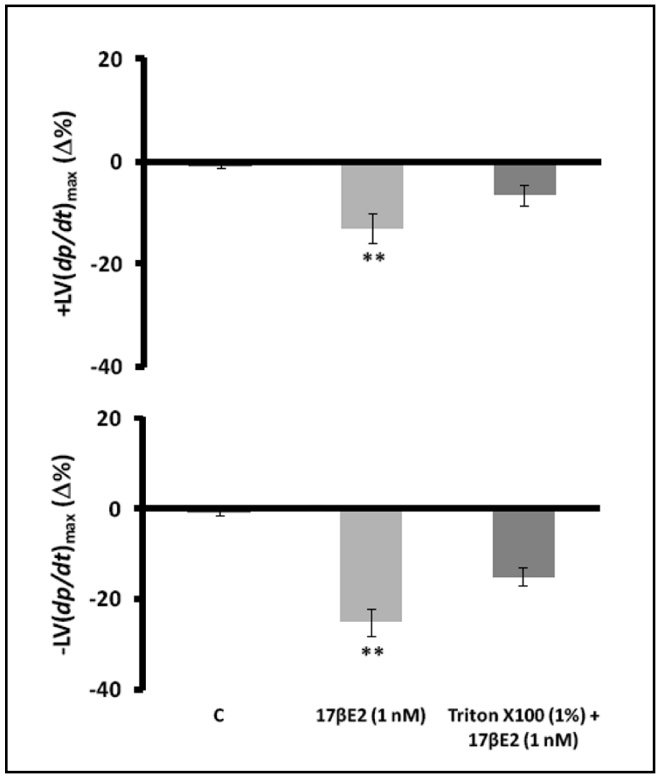

Fig. 5. Effects of 17βE2 before and after treatment with TritonX100 (1%) on $+(LVdP/dt)_{max}$ and $-(LVdP/dt)_{max}$ in the isolated and Langendorff perfused rat heart. Percentage changes were evaluated as means±SE of 6 experiments for each group. Significance of difference (one-way ANOVA) from control values of 17βE2 vs. Krebs-Henseleit solution (KHs): *P<0.05, **P<0.01. Comparison between groups treated with 17βE2 vs. TritonX100: §- p<0.05.

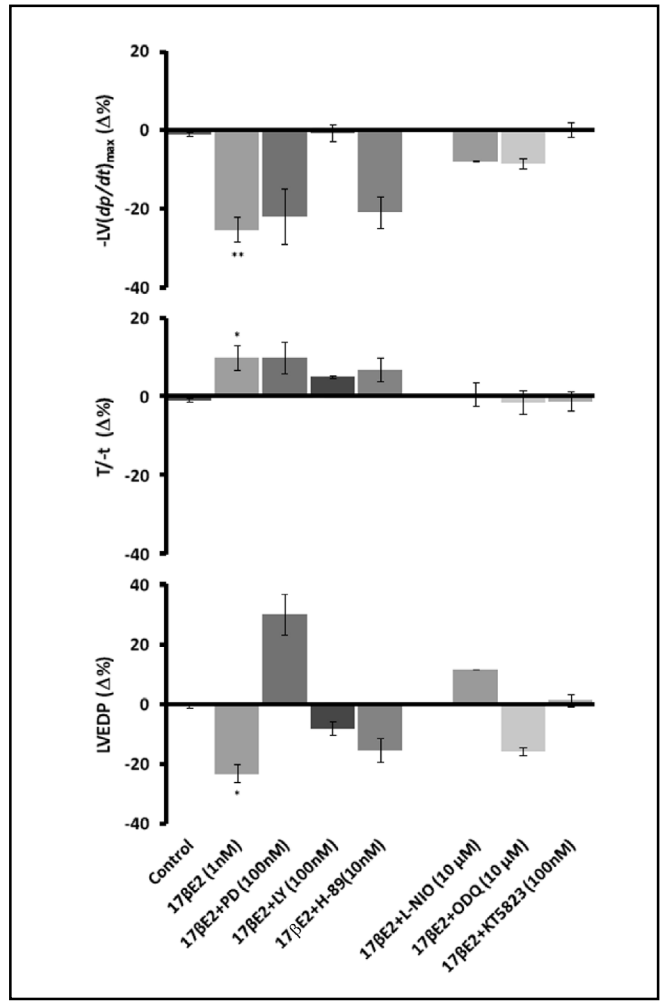

Fig. 6. Effects of 17βE2 before and after treatment with PD98095 (100 nM), LY (100 nM) and H-89 (10 nM), L-NIO (10 μM) or ODQ (10 μM), KT5823 (100 nM) on $-(LVdP/dt)_{max}$, T/-t, LVEDP on the isolated and Langendorff perfused rat heart preparation. Percentage changes were evaluated as means ±SE of 10 experiments for each group. Significance of difference (one-way ANOVA) from control values of 17βE2 alone vs. Krebs-Henseleit solution (KHs): *- P<0.05, **- P<0.01. Comparison between groups of 17βE2 alone vs. antagonist: §- p<0.05.

lusitropism was assessed by using specific inhibitors that, used alone, did not significantly modify the cardiac performance. Application of LY (100 nM), selective PI3K blocker, abolished the effects of 17βE2 on ventricular relaxation (Fig. 6), indicating the involvement of PI3K. In contrast, pretreatment with either PD98089 (specific ERK1/2 inhibitor) or H89 (specific PKA inhibitor) revealed that the cardiac relaxation induced by 17βE2 (Fig. 6) is independent from ERK1/2 or PKA.

Involvement of eNOS-NO-sGMP-PKG pathway

To assess the contribution of eNOS-NO-sGMP-PKG cascade to the negative lusitropic action of 17βE2, the effects of the hormone were evaluated in the presence of L-NIO (10 μM), ODQ (10 μM) and KT5823 (100 nM), inhibitors of eNOS, sGMP, and PKG, respectively. As shown in Fig. 6, exposure to these inhibitors prevented the 17βE2-induced reduction of relaxation (Fig. 6).

PLN phosphorylation

Negative lusitropism associates with phosphorylation of various cellular proteins, including the SERCA2a regulator PLN [25]. To assess the contribution of PLN to the negative lusitropism induced by 17βE2, Western Blot analyses were performed in cardiac tissue homogenate and cardiomyocyte lysates treated with the hormone. Fig. 7a shows that, in both hearts and cardiomyocytes, 17βE2 induced a reduction of PLN phosphorylation at ^{16}Ser but not at ^{17}Thr.

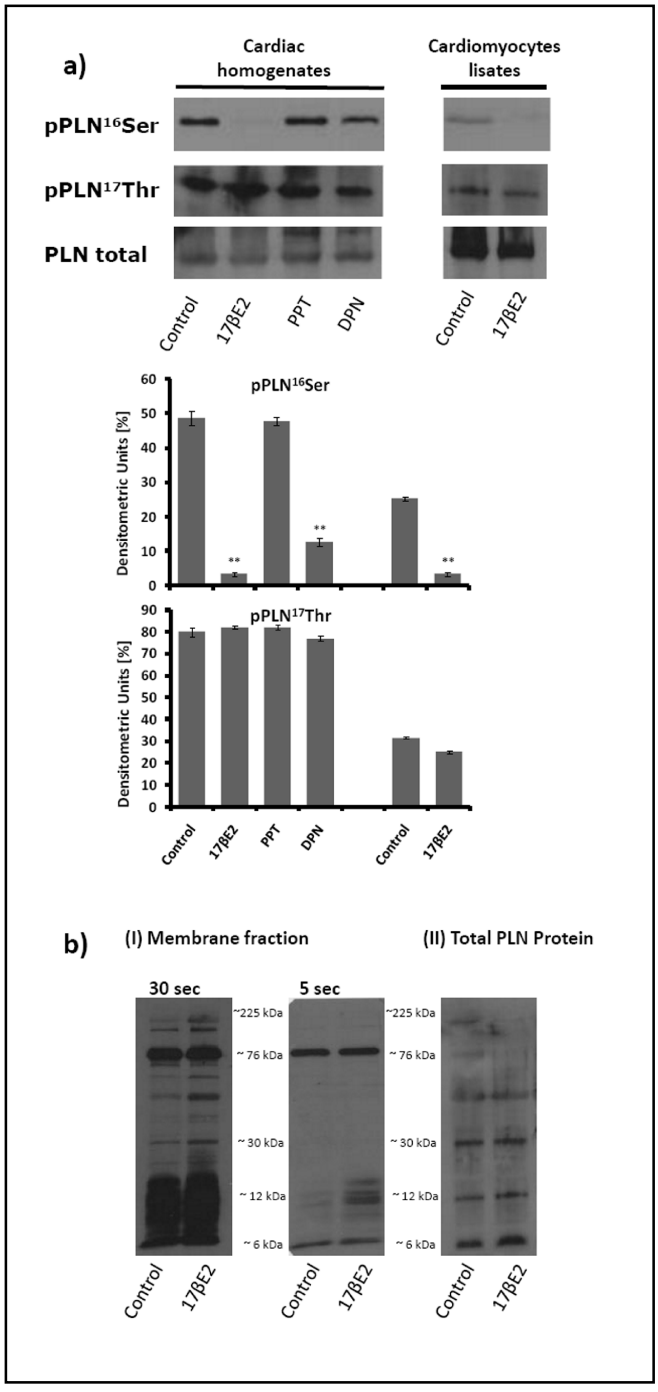

Fig. 7. a) Immunoblots of pPLN[16]Ser, pPLN[17]Thr and PLN total in control, 17βE2 treated-, PPT treated-, DPN treated-hearts (cardiac homogenates). Immunoblots of pPLN[16]Ser, pPLN[17]Thr and PLN total in control and 17βE2 treated- cells (cardiomyocytes lysates). Percentage changes were evaluated as means±SE of 6 experiments for each group. Significance of difference from control values (one-way ANOVA): *P<0.05, **P<0.01. b) Biotin switch assay of S-nitrosylated proteins (I) (exposed for 30 sec and 5 sec) and Western Blotting of total PLN (II) [monomer (6 kDa), dimer (12 kDa) and pentamer (30 kDa)] in the membrane fraction of homogenates from control and 17βE2-treated hearts.

To evaluate whether ERα and ERβ are involved in 17βE2-mediated reduction of PLN phosphorylation, Western Blotting analysis was carried out on cardiac preparations treated with PPT and DPN, specific ERα and ERβ agonists, respectively. We found that PLN phosphorylation was reduced only in heart treated with DPN (Fig. 7a).

Protein S-nitrosylation

S-nitrosylation of cardiac proteins has been recently reported to play a role in controlling heart performance [26]. By using the biotin switch method we evaluated whether 17βE2 affects S-nitrosylation of cardiac proteins. As shown in Fig. 7b (I), 17βE2-treated hearts showed an increased S-nitrosylation at the level of the membrane fraction, particularly evident at higher and lower molecular weights. Blots exposed to different times (5 sec and 30 sec) provided a better discrimination of the different bands. The shorter exposition time allowed to visualize the bands at low molecular weights. Western Blotting identification of the putative S-nitrosylated proteins with anti-PLN antibody revealed three bands corresponding to PLN monomer (6 kDa), dimer (12kDa) and pentamer (30 kDa) [Fig. 7b (II)].

Discussion

A major finding from this study is that, on the isolated and Langendorff perfused rat heart, under unstimulated conditions 17βE2 depresses ventricular relaxation in a dose-dependent manner. Being the hormone able to elicit also a basal negative inotropic effect [7], we propose 17βE2 as an important inhibitor of global myocardial mechanical performance. This negative lusitropic effect is mediated by the selective activation of the ERβ subtype, and requires the recruitment of the PI3K, eNOS-NO-sGMP-PKG pathways. It is also associated with an enhanced PLN phosphorylation and an increased S-nitrosylation of membrane proteins.

17βE2-induced negative lusitropic effects, showed by the reduction of the maximal rate of both the left ventricular pressure decline [$-LV(dp/dt)_{max}$], and by increments of T/-t and τ, were evident starting from picomolar concentrations, suggesting 17βE2 as a potent endogenous negative lusitropic substance. Notably, 17βE2 significantly reduced LVEDP, an important marker of diastolic function. Since LVEDP elevations are considered indexes of cardiac dysfunction, as observed in ischemia/reperfusion [27], we speculate that the cardiodepression

elicited by 17βE2 is not detrimental for the heart. This agrees with the cardioprotection elicited by nanomolar estrogen doses in male rats subject to ischemia/reperfusion [28]. Also in women it has been reported by clinical studies that endogenous estrogens (as in premenopausal period) contribute to reduce the risk for cardiovascular events [29]. Contrarily, two random trials failed to demonstrate cardioprotection following administration of exogenous estrogens in old women with or without established coronary vascular disease [30, 31]. Based on these observations, our data are of relevance in order to establish in male the benefits on cardiovascular function and possible therapeutic estrogen application.

We previously demonstrated that 17βE2-dependent negative inotropism is mediated by a cross-talk between the different cardiac ERs subtypes [7]. We showed here that the lusitropic action elicited by 17βE2 is mainly mediated by ERβ stimulation. In fact, only ERβ activation by DPN is able to mimic the effects elicited by 17βE2 on relaxation. The pathophysiological significance of ERβ in the heart is not fully elucidated, although accumulating evidence indicates this receptor be able to activate cardiac non genomic signaling [32, 33]. So far, ERβ was mainly found to elicit cardioprotection, since ERβ deficiency exacerbates postischemic myocardial dysfunction in hearts with hypercontractile condition [34], while activation of this receptor subtype restored 17βE2-mediated cardiac protection in ovariectomizated females under conditions of enhanced contractility subsequent to ischemia/reperfusion injury [35].

Interestingly, we observed that the negative modulation of ventricular relaxation of 17βE2 was abolished by inhibiting the eNOS-NO-cGMP-PKG pathway. This is in agreement with the role played by the NO-dependent downstream cascade in the negative lusitropism induced by several endogenous substances, such as the Chromogranin A-derived peptides Vasostatin and Catestatin [10-12]. These observations are in line with the accepted view that the cardiac NO system represents a convergence point for multiple negative signaling molecules which act in concert to reduce the detrimental effects of excessive stimulation, as in the case of the anti-adrenergic Chromogranin A-drived peptides. This may be of relevance since it increases the robustness and flexibility of the neuroendocrine control of the heart [36].

Many observations call for a role of NO in mediating the cardiac effects of 17βE2 [37, 38, 7]. In particular, an increased production of NO is observed in cardiomyocytes after ERβ activation [39], thus suggesting that this receptor subtype is responsible for the autocrine production of the gas. In addition to the autocrine release, a notable contribution to the nitrergic modulation of the heart is also provided by the paracrine production of NO which is generated by eNOS in the coronary endothelium, and that elicits important effects on relaxation [40]. Since functional ERs are expressed in vascular endothelial cells [41], it is presumable that the observed attenuation of the 17βE2-dependent lusitropic effect may be induced by the impaired production of paracrine NO subsequent to endothelial disruption by Triton X-100.

It is well known that E2-induced NO production triggers the activation of the cGMP pathway [42]. In the present work we found that the 17βE2-dependent negative lusitropism involves cGMP and its associated kinase (PKG).

Contrarily to these observations, cGMP was found to be only partially responsible for the 17βE2-dependent negative inotropism [7]. These discrepancies may be explained by the spatio-temporal compartmentalization of NO production by the differently localized NOS isoforms [43, 44], as well as by the NO-mediated activation of cGMP-dependent and/or cGMP-independent signaling pathways which target individual ECC proteins in the cardiac myocyte [45].

17βE2-induced negative lusitropism was abolished by LY, suggesting a PI3K-dependent mechanism. The PI3K/Akt pathway is involved in the acute nongenomic actions of 17βE2 in various cells types [32], as well as in many responses elicited by this hormone at the level of cardiac myocytes [46]. Its implication in 17βE2 effects has been related especially to heart protection against ischemia [47]. Upregulation of PI3K/Akt by 17βE2 results in eNOS activation via a transcription-independent mechanism [48, 49] which is mainly associated with ERα activation [32]. So far, little information exists regarding its possible involvement also in ERβ -mediated nongenomic effects [33]. However, recent data support the possibility that also ERβ signals via PI3K/Akt, since this enzymatic cascade was found to play a role in the cardioprotection elicited by 17βE2 in cardiac H9c2 cells against oxidative stress-induced apoptosis [50].

A major mechanism to initiate myocardial relaxation is the uptake of Ca^{2+} into SR through the SR Ca^{2+}-ATPase (SERCA2a). Under normal conditions, SERCA2a is controlled by its alternate association/dissociation with the regulatory protein PLN [51]. Dephosphorylated PLN inhibits SERCA2a and this induces more Ca^{2+} to be available for the contractile apparatus, thus decelerating relaxation. When PLN is phosphorylated, this inhibition

is relieved and Ca^{2+} is actively pumped into the SR and relaxation is faster [51]. Consistent with this mechanism, we found that the level of PLN^{16}Ser phosphorylation decreases under 17βE2 stimulation both in perfused hearts and in isolated myocytes. These data agree with the decreased PLN^{16}Ser phosphorylation elicited by other negative lusitropic substances, such as the Chromogranin-A-derived Catestatin [10]. Consistent with the role played by ERβ in 17βE2-dependent effects, we observed that also ERβ activation reduces PLN^{16}Ser phosphorylation. Of note, after 17βE2 exposure we didn't observe any change in PLN phosphorylation at ^{17}Thr, a modification which is mainly observed under pathological conditions [52].

S-nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine, has been recently recognized to be important in regulating protein function [13]. This is particularly relevant in the heart, in which several proteins of critical significance were identified as potential targets for S-nitrosylation [53]. We found here that 17βE2 increases the level of S-nitrosylated cardiac membrane proteins and this agrees with recent findings showing that 17βE2 promotes protein S-nitrosylation [54]. By Western Blotting we identified the putative target for S-nitrosylation, revealing three bands corresponding to the molecular weights of PLN monomer, dimer and pentamer. Of note, the monomeric (active) PLN showed no changes in S-nitrosylation, while the pentameric (inactive) form resulted more nitrosylated [55]. These observations support the hypothesis that 17βE2 controls myocardial mechanical performance by inducing NO-dependent S-nitrosylation of specific proteins, such as PLN.

In conclusion, our current findings indicate that 17βE2 directly reduced cardiac relaxation in a concentration-dependent manner. This effect is mediated by PI3K, eNOS-NO-cGMP-PKG, and requires PLN activity is regulated by phosphorylation and S-nitrosylation. Since cardiac muscle relaxation substantially contributes to optimal cardiac performance by allowing the heart to return to precontractile conditions of length and force, thus regulating diastolic filling and ejection fraction [16 and references therein], the discovery of new endogenous negative lusitropic modulators may shed light on the mechanisms which contribute to cardiac pathologies characterized by an altered diastolic function. In particular, the finding that the 17βE2-dependent negative lusitropic effect involves selective ER-β activation may offer new perspectives to study putative estrogen-dependent therapeutic applications.

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