JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 641–646

Received December 04, 2008
Accepted February 10, 2009
DOI 10.1211/jpp/61.05.0013
ISSN 0022-3573

Research Paper

Endothelium-independent vasorelaxation by the selective alpha estrogen receptor agonist propyl pyrazole triol in rat aortic smooth muscle

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Abstract

Objectives This study investigated the signalling mechanism of the relaxant responses to the estrogen receptor alpha (ERα) agonist PPT (propyl pyrazole triol) in endothelium-denuded rat aortic rings.

Methods Several compounds, including protein kinase G (PKG) inhibitors and potassium channel inhibitors, were tested against PPT-dependent rat aortic relaxation. Cyclic GMP and cytosolic calcium responses to PPT in isolated aortic smooth muscle were investigated in parallel.

Key findings PPT vasorelaxation was largely reduced by the selective ERα antagonist methyl-piperidinopyrazole (MPP; –91.6 ± 2.5%), by the selective PKG inhibitor Rp-8-Br-cGMP (–78.6 ± 4.9%), by the specific soluble guanylyl cyclase inhibitor ODQ (1*H*-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one; –85.3 ± 5.2%) and to a lesser extent by the selective BK_{Ca} (large-conductance calcium- and voltage-activated potassium channel) inhibitor iberiotoxin (–59.3%), the selective IK_{Ca} (intermediate-conductance calcium-activated potassium channel) inhibitor TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; –50.7%) and the voltage-gated potassium channel inhibitor 4-aminopyridine (–40.8%). In isolated aortic smooth muscle, PPT strongly enhanced the cyclic GMP content (+144%) and Rp-8-Br-cGMP largely reduced the PPT-dependent calcium signal (–80.8%).

Conclusions ERα receptor stimulation in rat aortic smooth muscle evokes a PKG-signalling pathway, likely triggering relaxation by BK_{Ca} and IK_{Ca} channel opening.

Keywords estrogen; PPT; protein kinase G; rat aorta; smooth muscle

Introduction

Nongenomic vasorelaxation seems to be one important mechanism explaining the cardioprotective properties of estrogens.[1] In isolated rat aorta, 17β-estradiol has an endothelium-independent, non-genomic vasorelaxant effect.[2,3] This effect is mediated by inhibition of calcium influx through both L-type and non-L-type calcium channels, but their signalling mechanism is unknown.[3]

The characterization of the pharmacological actions of estrogen receptor (ER) subtypes was facilitated by the introduction of the selective ERα agonist propyl pyrazole triol (PPT; Figure 1).[4,5] PPT has a 410-fold binding affinity preference for ERα and can fully activate genes through ERα, whereas there is no gene activation through ERβ.[4] PPT was found to relax endothelium-denuded rat aorta.[6] Therefore, we decided to examine the involvement of potassium channels in PPT relaxation of rat aortic smooth muscle. As Bolego *et al.*[7] pointed out, the importance of this kind of study resides in the fact that PPT or other tissue-selective ER modulators may be safer agents than endogenous estrogens for cardiovascular disease.

Materials and Methods

Compounds

PPT was obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Other compounds were from Sigma Chemical Co. (St Louis, MO, USA). Concentrated solutions of compounds in

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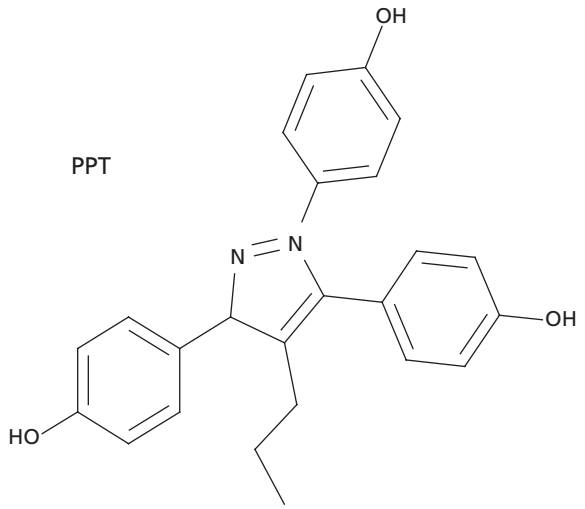

Figure 1 Chemical structure of the estrogen receptor α agonist PPT (propyl pyrazole triol).

dimethyl sulfoxide (DMSO) were prepared on the day of the experiment and diluted in incubation media, provided that the final DMSO concentration had no effect *per se* on tissue contractility.

Isolated rat aortic rings

Experiments were performed according to the European Community guidelines for animal ethical care and the Guide for care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). The study was approved by the ethical committee of the Department of Physiology and Pharmacology (School of Medicine, University of Zaragoza, Spain).

Relaxant responses of isolated rat aortic rings were investigated by using methods described in detail in previous publications.[8,9] Briefly, male Wistar rats, 250–300 g, were deprived of food the evening and the morning before the experiment. Similarly to several other investigators,[3,6,10–13] we used male rats due to their lower interindividual variability in estrogen nongenomic responses. Anaesthesia was induced with pentobarbital sodium (60 mg/kg, i.p.). After cervical dislocation, their thoracic aortas were immediately removed and carefully cleaned. The endothelium was removed by gently rubbing the intimal surface of the aorta with a small wooden stick. The thoracic aorta was cut into rings (3 mm long) that were individually placed between platinum hooks in 5 ml Krebs solution maintained at 37°C and gassed with 95% O_2–5% CO_2.

The Krebs medium contained (in mM): NaCl 118, $NaHCO_3$ 25, $CaCl_2$ 1.25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.5. To suppress basal tone, isoproterenol 1 μM was added to the incubation media for 10 min. An initial load of 1 g was applied to the preparations. Then, the preparations were washed three times and maintained throughout a 40-min equilibration period during which the incubation medium was renewed every 20 min. Tension was recorded on a MacLab (Analog Digital Instruments, Castle Hill, Australia) via Dynamometers Pioden Control UF1. Then, aortic rings were contracted with phenylephrine 1 μM or KCl 80 mM (NaCl was isotonically replaced with KCl). Phenylephrine 1 μM evoked maximal contractile responses. Fifteen minutes later, different compounds at a single concentration were tested against PPT vasorelaxation (see legends to figures). The concentration of the tested compounds was selected in control experiments on the basis of: (1) having no effect *per se* on vascular tone and (2) being active on the desired target as reported in the literature. Fifteen minutes later, PPT was added at a submaximum concentration. An absence of functional endothelium was indicated by the failure of the preparation precontracted with a submaximum concentration of phenylephrine to relax to acetylcholine (1 μM).

Cyclic GMP (cGMP) determination

Endothelium-denuded aortic segments were separated from the adventitia. The aortic segments were frozen with liquid nitrogen, then homogenized with 0.1 M HCl, centrifuged and the supernatants were used to determine cGMP by competitive ELISA assay (Thermoscientific, Waltham, MA, USA). Protein was determined by using a BCA protein assay kit.

Isolation of aortic smooth muscle cells

Endothelium-denuded aortic segments were separated from the adventitia. The muscular layer was dissociated and digested with collagenase type II in phosphate-buffered saline (PBS) for 8 min at 37°C. Then, samples were washed once with Krebs medium with fetal calf serum (FCS) and twice with the Krebs medium alone, filtered through a 50-micron mesh and finally suspended in Krebs medium with 10 mM Hepes pH 7.4 (without red phenol). The Krebs medium had the following composition (in mM): NaCl 130, $CaCl_2$ 1.25, $MgSO_4$ 1.2, Na_2PO_4H 1.2, glucose 11.5, KCl 4.7, HEPES 10 mM (pH 7.4).

Cytosolic calcium

Free cytosolic calcium in isolated aortic smooth muscle cells was measured by using a previously published method.[14] Briefly, cells were loaded for 15 min with Fluo 3-AM (Invitrogen, Carlsbad, CA, USA). Fluorescence quantification was performed by using flow cytometry (Epics Elite; Beckman Coulter, Fullerton, CA, USA) with a 70 μM flow cell at 37°C. Cells were excited with an argon laser at 488 nm and the emitted fluorescence was 525 ± 25 nm. Fluorescence readings were expressed in arbitrary units.

Statistical analysis

Values are given as mean ± SD. Concentration–response curves of compounds were fitted by linear regression analysis of the straight portions of the vasorelaxant responses. The concentration of compound antagonizing 50% of the maximal responses to vasorelaxant agonists (IC50) was calculated for each experiment. Statistical differences between mean values were determined by using an unpaired Student's *t*-test. Multiple measurement comparison was performed by using an analysis of variance program followed by a Bonferroni–Dunn test. Factorial analysis was also performed by using analysis of variance. $P < 0.05$ indicated statistical significance.

Results

PPT was tested at multiple concentrations for its vasorelaxant properties in phenylephrine-precontracted rat aortic rings, with and without endothelium ($n = 5$). Figure 2 shows that endothelial removal was without effect on PPT vasorelaxant potency. The PPT IC50, calculated from the concentration–response curves, was 39.8 ± 6.2 μM and 35.2 ± 5.8 μM in aortic rings with and without endothelium, respectively. Therefore, all subsequent experiments were performed in endothelium-denuded aortic rings. The inset to Figure 2 shows that PPT relaxed aortic rings precontracted with phenylephrine or KCl with similar potency.

Effect of estrogen receptor antagonists

PPT interaction with estrogen receptors was investigated by using the selective ERα antagonist methyl-piperidinopyrazole (MPP)[15] against the vasorelaxant responses to submaximal concentrations of PPT (50 μM) (Figure 3). MPP was used at 1 μM, because: (1) this concentration was previously found to selectively block ERα stimulation of pS2 mRNA in MCF-7 breast cancer cells[15] and (2) MPP concentrations higher than 1 μM provoke vasorelaxation. Figure 3 shows that MPP 1 μM blocked PPT vasorelaxation by 91.6 ± 2.4% ($n = 6$).

Other estrogen receptor antagonists (tamoxifen, 4-OH-tamoxifen and ICI 182,780) had a vasorelaxant effect by themselves at high concentrations (Figure 3, inset). Therefore, a maximal non-vasorelaxant 4-OH-tamoxifen concentration of 100 nM was selected to be tested for PPT antagonistic properties. Figure 3 shows that 100 nM 4-OH-tamoxifen reduced the effect of PPT by 71.5%.

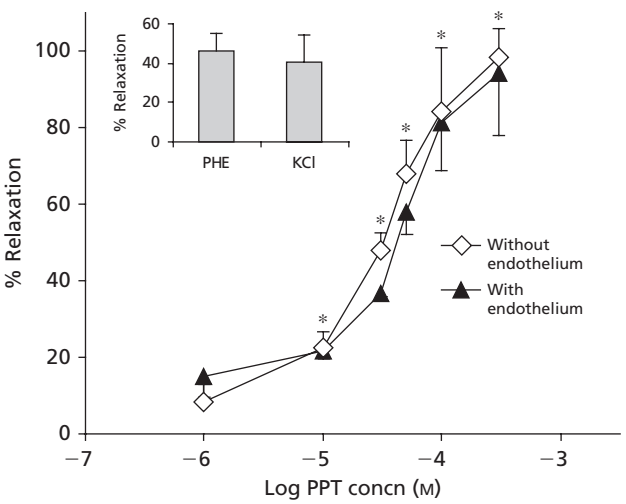

Figure 2 Relaxation by PPT of phenylephrine-precontracted rat aortic rings. Values are given as mean ± SD, $n = 5$ for condition. No statistically significant differences were found between values with and without endothelium (analysis of variance multiple measurement comparison, followed by the Bonferroni–Dunn test). *$P < 0.05$ compared with control values (one-way analysis of variance followed by Tukey's test). Inset: % relaxation by PPT 50 μM against contractions induced by phenylephrine 1 μM (PHE) or KCl 80 mM (KCl). Differences were statistically non-significant (unpaired Student's t-test).

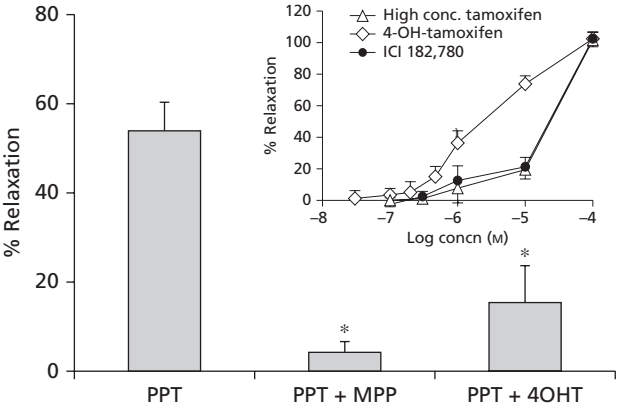

Figure 3 Antagonism of PPT vasorelaxation by MPP and 4-OH-tamoxifen (4OHT). PPT (50 μM), MPP (1 μM) and 4-OH-tamoxifen (100 nM) were tested in endothelium-denuded rat aorta precontracted with phenylephrine. Values are given as mean ± SD, $n = 6$. *$P < 0.01$ compared with PPT alone (one-way analysis of variance, followed by bilateral Dunnett's t-test). Inset: Relaxation of phenylephrine-precontracted rat aortic rings by high concentrations of tamoxifen, 4-OH-tamoxifen and ICI 182,780. Relaxation values higher than 20% were statistically significant with respect to control values (one-way analysis of variance followed by Tukey's test).

Effect of potassium channel inhibitors

The selective large-conductance calcium- and voltage-activated potassium channel (BK_{Ca}) inhibitor iberiotoxin reduced the vasorelaxant responses to PPT, but to a lesser extent than tamoxifen or Rp-8-Br-cGMP (–59.3%; Figure 4).

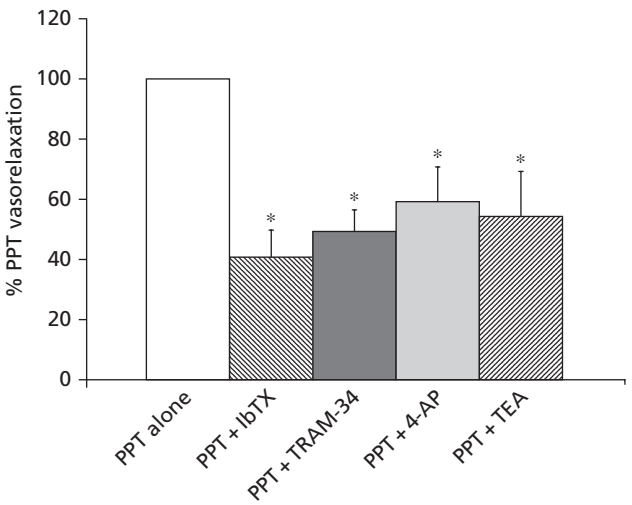

Figure 4 Effect of the selective BK_{Ca} inhibitor iberiotoxin and the selective IK_{Ca} inhibitor TRAM-34 on the vasorelaxant responses of rat aorta to PPT. Tested compounds were PPT alone (50 μM), PPT + IbTX (iberiotoxin 30 nM), PPT + TRAM-34 (10 μM), PPT + 4-AP (4-aminopyridine 1 mM) and PPT + TEA (tetraethyl-ammonium 2 mM). Compounds were tested in endothelium-denuded rat aorta precontracted with phenylephrine. Values are given as mean ± SD, $n = 6$ for condition. *$P < 0.05$ compared with PPT alone (one-way analysis of variance, followed by bilateral Dunnett's t-test). PPT alone (50 μM) relaxed 48.1 ± 7.1% of phenylephrine-dependent contraction.

PPT vasorelaxation was also partially blocked by: (1) the selective inhibitor of calcium-activated potassium channels of intermediate conductance (IK_{Ca}) TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; –50.7%); (2) the voltage-gated potassium channel (K_v) inhibitor 4-aminopyridine (–40.8%) and (3) the non-specific potassium channel inhibitor TEA (tetraethyl-ammonium; –45.7%) (Figure 4). Conversely, the ATP-sensitive potassium channel (K_{ATP}) inhibitor glibenclamide 10 μM was unable to significantly reduce PPT vasorelaxation (–5.2 ± 7.1%, $n = 5$).

Effect of PKG, PKA and guanylyl cyclase inhibitors

Vasorelaxant responses to submaximal concentrations of PPT (50 μM) were investigated in aortic rings preincubated with the protein-kinase G (PKG) inhibitor Rp-8-Br-cGMP (10 μM), the protein kinase A (PKA) inhibitor H-89 (*N*-[2-bromocynnamyl (amino)ethyl]-5-isoquinoline sulfonamide; 200 nM) and the specific soluble guanylyl cyclase inhibitor ODQ (1*H*-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one; 1 μM) ($n = 6$ per condition). PPT vasorelaxation was largely reduced (–78.6%) by preincubation with Rp-8-Br-cGMP or ODQ (–85.3%) (Figure 5). Conversely, H-89 was unable to modify PPT vasorelaxation (–0.8 ± 8.3%) (Figure 4).

cGMP content

The cGMP content in isolated aortic smooth muscle was measured as described in the Methods section. Table 1 shows that PPT 100 μM increased the cGMP content from a basal value of 0.84 ± 0.28 pmol/mg protein up to 2.05 ± 0.3 pmol/mg protein ($n = 4$). For comparison, nitroprusside 10 μM increased cGMP up to 3.60 ± 0.24 pM/mg protein.

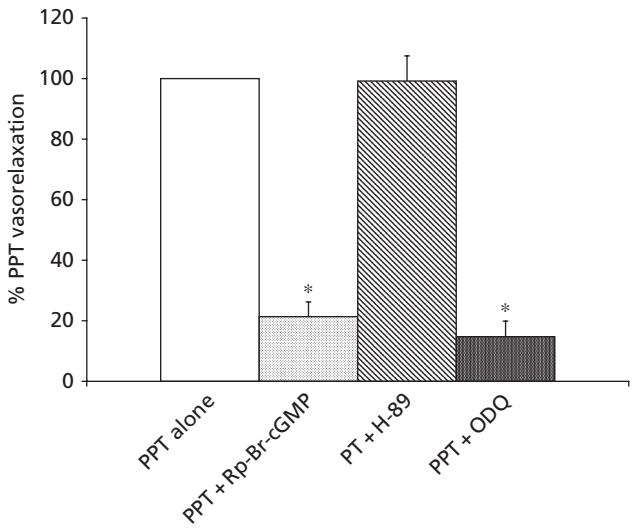

Figure 5 Effect of the selective PKG inhibitor Rp-8-Br-cGMP and the selective PKA inhibitor H-89 on the vasorelaxant responses of rat aorta to PPT. Tested compounds were PPT alone (50 μM), PPT + Rp-Br-cGMP (10 μM), PPT + H-89 (200 nM) and PPT + ODQ (1 μM). Compounds were tested in endothelium-denuded rat aorta precontracted with phenylephrine. Values are given as mean ± SD, $n = 6$ for condition. *$P < 0.01$ compared with PPT alone (one-way analysis of variance, followed by bilateral Dunnett's *t*-test); *$P < 0.05$ compared with PPT alone (non-paired Student's t-test).

Table 1 cGMP content in rat isolated aortic smooth muscle cells exposed to different agents

Compound	cGMP content (pmol/mg protein)
Control	0.84 ± 0.28
PPT 100 μM	2.05 ± 0.38*
Nitroprusside 10 μM	3.60 ± 0.24*

Values are given as mean ± SD, $n = 4$. *$P < 0.01$ versus control (one-way analysis of variance, followed by bilateral Dunnett's *t*-test).

Cytosolic calcium

Free cytosolic calcium in isolated aortic smooth muscle cells was measured as described in the Methods section. Phenylephrine 2 μM induced a 20.3 ± 2.5% increase in the fluorescence signal. In cells preincubated with 2 μM phenylephrine, PPT (50 μM) reduced the phenylephrine-induced calcium signal by 70.6 ± 9.95%, an effect largely antagonized (–80.8%) by preincubation with the PKG inhibitor Rp-8-Br-cGMP (10 μM) (Figure 6).

Discussion

In endothelium-denuded rat aorta, the relaxant responses to the ERα agonist PPT were largely reduced by the selective ERα antagonist MPP and partially reduced by the BK_{Ca} inhibitor iberiotoxin, the selective IK_{Ca} inhibitor TRAM-34 and the voltage-gated potassium channel inhibitor 4-aminopyridine. The PKG inhibitor Rp-8-Br-cGMP and the specific soluble guanylyl cyclase inhibitor ODQ, but not the PKA inhibitor H-89, largely reduced PPT vasorelaxation. In isolated aortic smooth muscle, PPT strongly enhanced the cGMP content and Rp-8-Br-cGMP largely reduced the PPT-dependent calcium signal. These results clearly show that ERα stimulation by PPT triggers a PKG-signalling pathway by acting directly in rat aortic smooth muscle.

Estrogen is usually presumed to dilate arteries by stimulating nitric oxide (NO) synthase activity in endothelial cells.[16] In coronary arteries, ERα stimulation evokes acute

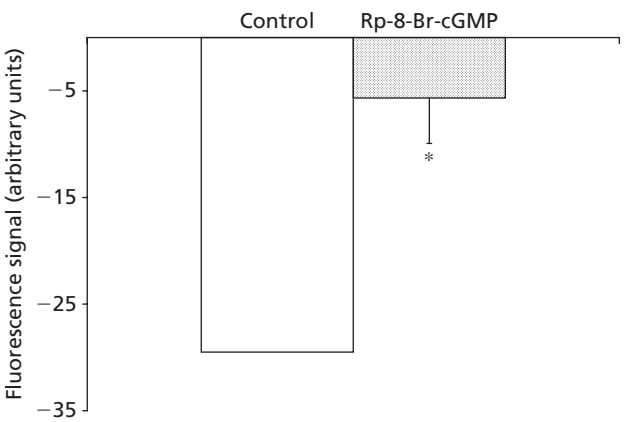

Figure 6 Calcium signal responses to PPT (50 μM) in isolated aortic smooth muscle cells, and their inhibition by the PKG inhibitor Rp-8-Br-cGMP (10 μM). Values are given as mean ± SD, $n = 6$. *$P < 0.05$ (non-paired Student's t-test).

non-genomic vasorelaxation via NO synthase stimulation in endothelial cells[13,17] and PKG activation of the BK_{Ca} channel in vascular smooth muscle cells, with cell membrane hyperpolarization and reduced cellular excitability.[18] On the other hand, human vascular smooth muscle expresses aromatase,[19] indicating a possible direct action of locally produced estrogen in an autocrine or paracrine manner, with possible cross-talk between smooth muscle and endothelial cells.[19] Moreover, Han *et al.*[18] found that ERα receptor stimulation can directly evoke PKG-dependent responses in human coronary artery smooth muscle, mediated by BK_{Ca} channel opening (for review see Ghatta *et al.*).[20] This response to estrogen appears to involve the NO/cGMP signalling system in coronary smooth muscle.[21]

In rat aorta, iberiotoxin only blocked 59.3% of PPT-mediated relaxation. Moreover, PPT vasorelaxation was also partially blocked by TRAM-34, a selective IK_{Ca} inhibitor, and by the K_v channel inhibitor 4-aminopyridine. The IK_{Ca} channel is expressed in vascular smooth muscle cells[22] but its regulation is poorly understood. In the rat middle cerebral artery, the IK_{Ca} channel does not appear to be regulated by the NO/cGMP signalling system.[23] Conversely, the mechanism by which acute NO stimulation may control arterial tone through ERK1/2 MAP-kinase dephosphorylation involves a complex of signalling cascades and interaction between cGMP, Kv.1.2 and SHP-1.[24]

The selective PKA inhibitor H-89 was unable to antagonize PPT-induced vasorelaxation. This differs from genistein, which exhibits selectivity for the ERβ receptor subtype[25] and seems to act via PKA signalling. Thus, genistein increases the cAMP content in rat aortic smooth muscle cells[26] and in porcine coronary arteries it was found that genistein-induced relaxation was abolished by the PKA inhibitor, Rp-8-Br-cAMPS.[27,28]

The vasorelaxation response of aortic rings to PPT was seen at micromolar concentration, thus casting doubt on the possible relevance for ERα activation in modulating smooth muscle activity. Estrogen relaxes endothelium-denuded human coronary arteries at concentrations within or near plasma levels (nanomolar).[29,30] However, non-genomic actions of estrogens are often seen at high (i.e. μM) concentrations.[5] In particular, micromolar concentrations of estrogen are required to relax isolated rat vessels,[2,3,5,11] including coronary arteries, whereas binding affinities are frequently seen in the nanomolar range. Moreover, PPT is not a natural substance, and it is difficult to directly extrapolate biologically relevant actions from its active concentration ranges. Therefore, the possible physiological relevance of our results is unclear.

Nongenomic vasorelaxation seems to be one important mechanism explaining the cardioprotective properties of estrogens.[1] Studies with knockout mice lacking ERα or ERβ suggested that ERα vasorelaxation mediates the protective actions of estrogen against coronary artery disease.[7] On the other hand, the localized expression of aromatase in human vascular smooth muscle[19] provides a mechanism for de-novo estrogen synthesis, from precursors such as testosterone, within the vascular wall. Unfortunately, our knowledge of the direct effects of estrogen on vascular smooth muscle cells remains rather limited. Our results in male rats thus deserve further investigation in female rats.

Conclusions

In conclusion, ERα-dependent relaxation of rat aortic smooth muscle evokes a PKG-signalling pathway, likely operating BK_{Ca} and IK_{Ca} channel opening. In addition to endothelium-independence, the involvement of the IK_{Ca} channel is another difference with the ERα signalling pathway in coronary arteries.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This investigation was supported by the Multidisciplinary Projects No. PM-031/2004 and PM045-2007 of Diputación General de Aragón (Spain).

Acknowledgements

We are greatly indebted to Laia Brines (School of Medicine, Zaragoza, Spain) for helping with the experiments, to Pedro Serrano (School of Medicine, Zaragoza, Spain) for helpful discussion and to William Crumb (New Orleans, LA, USA) for revising the English.

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