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Medroxyprogesterone acetate attenuates long-term effects of 17β-estradiol in coronary arteries from hyperlipidemic rabbits

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ABSTRACT

Objective: The progestin component in hormone replacement treatment may oppose the effects of estrogen on vascular function. This study examined the effect of long-term treatment with 17β-estradiol (E_2) alone and in combination with two progestins on K^+ and Ca^{2+}-mediated mechanisms in coronary arteries.

Methods: Watanabe heritable hyperlipidemic rabbits were treated orally with either E_2 (4 mg/day), medroxyprogesterone acetate (MPA) (10 mg/day), norethindrone acetate (NETA) (2 mg/day), E_2 + MPA, E_2 + NETA, or placebo for 16 weeks ($n = 10$ in each group). Coronary arteries were used for mRNA and myograph studies.

Results: E_2 increased vasodilatation induced by sodium nitroprusside and decreased vasocontraction induced by potassium. The first but not the latter response was opposed by MPA. The combination of MPA and E_2, but neither compound alone enhanced nimodipine-induced vasodilatation and increased the expression of L-type voltage-gated Ca^{2+} channel mRNA. NETA had no opposing effects. Hormone treatment did not affect large-conductance Ca^{2+} activated or ATP-sensitive K^+ channels or cGMP-dependent protein kinase mRNA expression. Hyperlipidemia had no effect on vascular reactivity.

Conclusion: When E_2 is administered with MPA, effects of E_2 on nitric oxide and Ca^{2+}-mediated vascular reactivity in rabbit coronary arteries are modulated. The results suggest that the progestin component in hormone replacement treatment may interfere with the supposed beneficial vascular effects of estrogen.

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1. Introduction

In contrast to numerous epidemiological and experimental studies, recent randomized clinical trials have refuted a beneficial vascular effect of long-term combined hormone replacement treatment (HRT) [1,2]. Progestin is added to estrogen to reduce the risk of uterine malignancy. However, progestin may oppose the effects of estrogen on vascular function [3,4]

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and different combinations of estrogen and progestin may affect vascular function differently [5]. The beneficial effects of estrogen on the vascular system include the ability to reduce vascular contractility [6,7] but the effects of combining progestins and estrogen on vascular function are poorly elucidated.

Acute vascular dilatation induced by 17β-estradiol (E_2) is mediated through vascular smooth muscle cells by stimulation of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) [8] through binding to its β subunit [9], by stimulation of ATP-sensitive K^+ (K_{ATP}) channels [10] and/or by the inhibition of L-type voltage-gated Ca^{2+} channels (VG-Ca^{2+}) [11–13]. Also, both acute and long-term E_2 treatment depresses vasocontraction by increasing the activity and expression of endothelial nitric oxide synthase (eNOS) [14] and hence the release of nitric oxide (NO) from the endothelium. NO induces relaxation of vascular smooth muscle cells, mainly through the activation of soluble guanylate cyclase which increases cyclic GMP levels, leading to activation of different types of K^+ channels including BK_{Ca} [15–17] and K_{ATP} [18,19] through cGMP-dependent protein kinase (PKG). Activation of K^+ channels leads to hyperpolarization of the vascular-smooth-muscle-cell membrane and inhibition of VG-Ca^{2+} which subsequently causes vessel relaxation. PKG and NO may also directly inhibit L-type Ca^{2+} channels [20,21]. Thus, K^+ and VG-Ca^{2+} channels are directly involved in the acute actions of estrogen and perhaps also in the long-term actions of estrogen through the modulation of these ion channels by the NO/cGMP/PKG pathway. The long-term effects of estrogen and the putative effect of combining estrogen with progestin on vascular function and gene expression involving K^+ and Ca^{2+} channels in vascular smooth muscle cells are not clear. To test the hypothesis that progestins might attenuate or oppose the beneficial effects of estrogen on vascular function, we investigated the long-term effects of 17β-estradiol alone or in combination with medroxyprogesterone acetate (MPA) or norethindrone acetate (NETA) in coronary arteries of hyperlipidemic rabbits. MPA, a 17α-hydroxyprogesterone derivative and NETA, a 19-nortestosterone derivative were chosen since they represent two important classes of synthetic progestins commonly applied in HRT [22].

2. Experimental

2.1. Animals and treatment

The animals were obtained from a breeding colony at the Danish Institute for Food and Veterinary Research (derived from a parent generation from FGJ Jansen, University of Nijmegen, The Netherlands with permission from Y. Watanabe). All study procedures were approved by The Animal Experiments Inspectorate (The Danish Ministry of Justice).

At 7 weeks of age (body weight > 900 g) 60 homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits were bilaterally ovariectomized under general anaesthesia (fentanyl 0.032 mg/kg + fluanisone 1 mg/kg + midazolam 0.5 mg/kg intravenously). After surgical recovery, the animals were allocated based on plasma cholesterol concentrations and body weight to six groups, which were randomly assigned to one of the following daily oral treatments for 16 ± 1 weeks: (1) 17β-estradiol (E_2) 4 mg, (2) E_2 4 mg + medroxyprogesterone 17-acetate (MPA) 10 mg, (3) E_2 4 mg + 19-norethindrone acetate (NETA) 2 mg, (4) MPA 10 mg, (5) NETA 2 mg and (6) placebo ($n = 10$ in each group). A group of age-matched placebo treated ovariectomized heterozygous WHHL rabbits was also studied ($n = 14$). The choice of dosage in this study was based on previous experiments in the rabbit showing serum concentrations of E_2 and MPA that are similar to those obtained in postmenopausal women [23–25]. The dose required to transform the endometrium to the secretory phase is five times higher for MPA than for NETA [22] and therefore NETA was administered as 2 mg/day. This dosage has previously been shown to have anti-proliferative effects in the rabbit uterus [26]. Treatments were given in a maize-based phytoestrogen low diet [27] (No. 2123, Altromin International, Germany). The test substances were added to a powdered formulation of the diet before being processed to pellets. The consumption of drug-containing diet and control diet was recorded daily. For all drug-containing diets the intake was >98% of the offered chow. Total plasma cholesterol was measured enzymatically (Cobas Mira S-analyzer, Roche Diagnostics Ltd.) at the initiation and termination of treatment. E_2 and NETA were obtained from Novo Nordisk A/S, Denmark and MPA from Sigma–Aldrich, Denmark. After 9 weeks of treatment fasting serum 17β-estradiol levels were measured by chromatography and radioimmunoassay (Statens Serum Institute) [28].

2.2. Tissue preparation

Rabbits were euthanized by phenobarbital (100 mg/kg intravenously) followed by exsanguination and perfusion with 400 mL 0.9% NaCl (4 °C). The intramural left anterior descending coronary artery (LAD) and the intramural right coronary artery (RCA) were isolated and freed of surrounding tissue under a microscope. During dissection, the tissue was immersed in an oxygenated (5% CO_2/95% O_2, 4 °C) physiological saline solution (PSS) with NaCl 119 mmol/L, $NaHCO_3$ 25 mmol/L, KCl 4.6 mmol/L, $MgSO_4$ 1.17 mmol/L, KH_2SO_4 1.18 mmol/L, $CaCl_2$ 1.5 mmol/L, EDTA 0.027 mmol/L, and glucose 5.5 mmol/L. The RCA was snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Ring segments from the LAD were prepared for myograph experiments. One segment from LAD was fixed, paraffin embedded and stained with elastic-van Gieson. Three serial cross sections from each animal were inspected for atherosclerosis in a light microscope. The weight of the uterus was recorded.

2.3. Myograph experiments

LAD ring segments were mounted in a myograph (Model 500A, Danish Myo Technology A/S, Aarhus, Denmark) in individual organ baths. After equilibration for 30 min in PSS at 37 °C the vessels were normalized, i.e. the internal circumference, L_1 was set to 90% of L_{100}, where L_{100} is the internal circumference the vessels would have in situ when relaxed and under a transmural pressure of 13.3 kPa (100 mmHg). At L_1 the force production is close to maximal [29]. After equi-

libration for 30 min vessel contraction was induced repeatedly by 124 mmol/L K^+ PSS until reproducible wall tensions were recorded. Segments producing a response less than 1 N/m were excluded. Before protocol specific experiments were performed vessels were allowed to equilibrate for 30 min.

In separate artery segments one of the following experimental protocols was employed: (1) Following pre-contraction with 50 mmol/L K^+ PSS cumulative concentration–response curves in response to sodium nitroprusside (SNP) (10^{-9} to 10^{-2} mol/L, Sigma–Aldrich) were obtained. (2) Cumulative concentration–response curves to KCl (K^+) were obtained (5.8–50 mmol/L). (3) To obtain cumulative concentration–response curves for calcium segments were washed three times with calcium-free PSS containing 0.5 mmol/L ethylene glycol-bis[β-aminoethyl ether]*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), after the last wash artery segments were incubated with phentolamine (3×10^{-6} mol/L, Sigma–Aldrich) for 20 min in order to inhibit the release of noradrenaline from perivascular nerves when depolarized by potassium. Cumulative concentration–response curves were then obtained by adding $CaCl_2$ (10^{-6} to 10^{-2} mol/L) to a Ca^{2+}-free, 124 mmol/L K^+ solution containing phentolamine (3×10^{-6} mol/L). After wash-out and equilibration in PSS for 30 min, the segments that were used for examination of the Ca-response were pre-contracted with 50 mmol/L K^+ PSS and cumulative concentration–response curves to nimodipine (10^{-10} to 10^{-7} mol/L, Sigma–Aldrich) were obtained. (4) Prior to pre-contraction with 50 mmol/L K^+ PSS cumulative concentration–response curve to acetylcholine (ACh 10^{-9} to 10^{-5} mol/L, Sigma–Aldrich) were obtained in the placebo treated animals.

The vessel response was expressed as active wall tension, δT (N/m). The theoretical maximum contractile and dilatory effect (E_{max}), given as N/m and % of precontraction, respectively, and the negative log of the molar concentration causing half-maximal response ($-\log[EC_{50}]$) were calculated by nonlinear regression analysis (Sigmoidal dose–response curve with variable Hill-slope) using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA). Full sigmoidal concentration–response curves were not obtainable for the acetylcholine- curve due to a biphasic response instead responses were read at 10^{-5} mol/L.

2.4. *RNA purification and cDNA amplification*

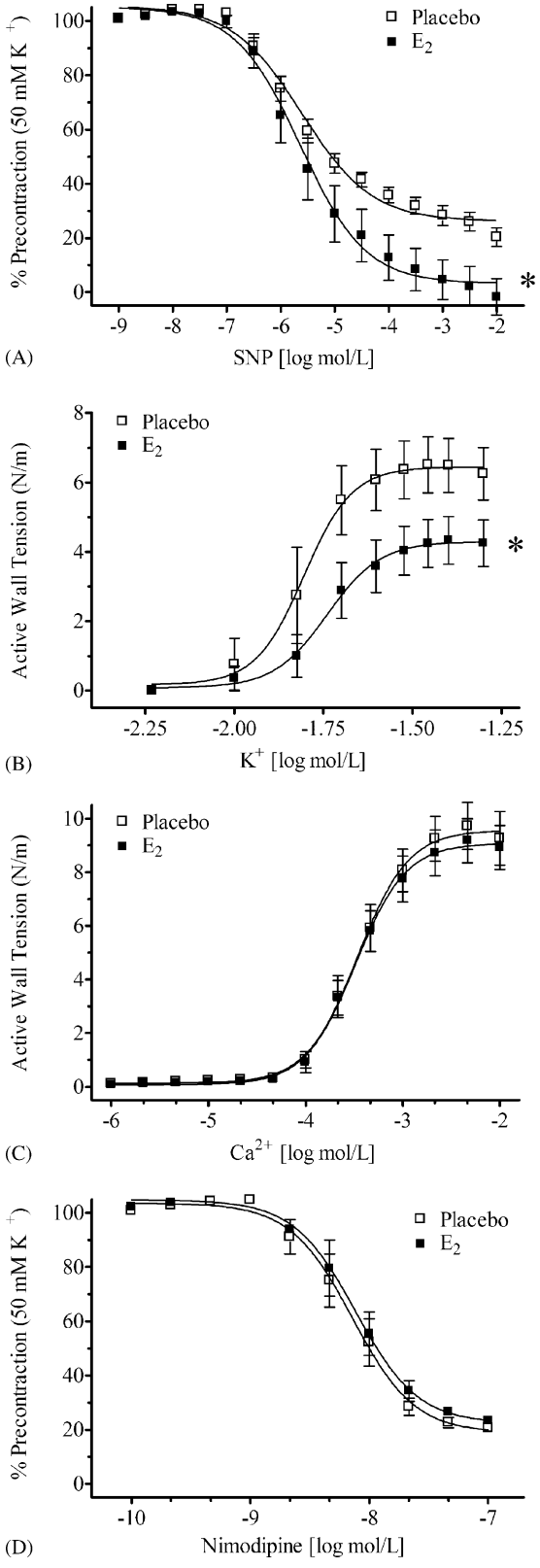
After tissue homogenization with a polypropylene micropestle, total cellular RNA was extracted from RCA with TRIzol® Reagent (Invitrogen, DK) with slight modifications from the manufacturer's instructions. Total RNA (1 μg) extracted from heterozygous rabbit aorta was used to generate a pool of cDNA for the generation of standard curves. First-strand cDNA was synthesized from total RNA using SuperScript™ II Reverse Transcriptase (Invitrogen) and oligo(dT)$_{12–18}$ as primer in a PTC-200™ Thermal cycler (MJ Research, Inc., USA). RNA purity and concentration were determined spectrophotometrically by A_{240}, A_{260} and A_{280} measurements. A no-RNA template control and a positive RNA control were included. RNA integrity was assured by seeing the 18S and 28S ribosomal bands after electrophoresis of total RNA on 1.4% agarose gels. Forward and reverse primers for amplification of L-type voltage-dependent calcium channel (VG-Ca^{2+}) (GenBank accession no. X55763.1), β subunit of large-conductance calcium-activated potassium channel (BK_{Ca}, β) (GenBank accession no. AB001934), Kir6.1 subunit of ATP-sensitive potassium channel ($K_{ATP,Kir\,6.1}$) (GenBank accession no. AY072063), cGMP-dependent protein kinase subtype 1 alpha (PKG1α) (GenBank accession no. AF076969), endothelial nitric oxide synthase (eNOS) (GenBank accession no. AY179960) and hypoxanthine-phosphoribosyl-transferase (HPRT) (GenBank accession no. AF020294) cDNAs were as follows: L-type VG-Ca^{2+} (5′-GAGTCTCCAGGTGGTCCTGA-3′ and 5′-CTGCTGGGACATCTGCTACA-3′), BK_{Ca} (5′-CCTCTACCAGAAAAGCGTGTG-3′ and 5′-ATGTAGGAGCACTGCTGATTCC-3′), K_{ATP} (5′-CACCAACGTCAGGTCTTTCA-3′ and 5′-TAGCACTGTGATGGCCAGAG-3′), PKG1α (5′-CAAGTTTGAAAGGTCCAAGGAT-3′ and 5′-ACTTCAACTTTCCCATCTTCCA-3′), eNOS (5′-CCTACAGGACCCAGGATGG-3′ and 5′-CAGCCCTTTGCTCTCAATGT-3′) and HPRT (5′-CTCAACCTTAACTGGAAAGAATGTC-3′ and

Table 1 – Basic characteristics

Treatment group	n_1	Body weight (kg)	Uterus (‰ BW)	Total cholesterol (mmol/L)	Vessel diameter (μm)							
					SNP	n_2	K	n_2	$CaCl_2$	n_2	Nimodipine	n_2
Placebo	10	2.7 (0.2)	0.1 (0.0)	22.7 (3.1)	731 (149)	9	766 (91)	8	706 (158)	9	710 (150)	10
E_2	10	2.8 (0.3)	2.7 (0.7)	25.0 (4.2)	668 (150)	9	706 (147)	9	702 (113)	10	702 (113)	10
E_2 + MPA	10	2.4 (0.1)‡	1.2 (0.2)	31.3 (6.6)**†	656 (132)	10	627 (124)	9	624 (111)	9	624 (111)	9
E_2 + NETA	10	2.7 (0.2)	2.4 (0.7)	29.9 (6.3)**†	734 (197)	9	754 (119)	10	775 (146)	10	784 (152)	9
MPA	10	2.4 (0.2)§	0.8 (0.2)	30.7 (7.4)	669 (147)	10	741 (209)	10	721 (133)	10	721 (133)	10
NETA	10	2.7 (0.2)	1.2 (0.2)	28.9 (5.4)	700 (172)	10	731 (138)	7	634 (138)	9	631 (147)	8
Placebo (hetero)	14	2.8 (0.1)	0.1 (0.0)	2.7 (0.7)**	679 (133)	13	730 (77)	9	681 (114)	13	685 (125)	11

Values are mean (S.D.), n_1, number of animals in each group, n_2, number of vessel segments in each protocol. E_2: 17β-estradiol; MPA: medroxyprogesterone acetate; NETA: norethindrone acetate; hetero = heterozygous WHHL rabbits treated with placebo. SNP: sodium nitroprusside; K^+: potassium; $CaCl_2$: calcium chloride. Body weight was lower in the MPA and E_2 + MPA treatment groups, vessel diameter did not differ between treatment groups (one-way ANOVA). Uterus weight varied according to treatment. The heterozygous placebo group had lower levels of total cholesterol compared to the homozygous placebo group. Addition of MPA and NETA increased the total cholesterol concentration. $^{‡}P < 0.01$ vs. placebo, E_2, E_2 + NETA, NETA, placebo (hetero); $^{§}P < 0.05$ vs. E_2, E_2 + NETA, NETA, placebo (hetero) (one-way ANOVA); $^{**}P < 0.01$ vs. placebo; $^{†}P < 0.05$ vs. E_2 (Student's *t*-test).

5′-CCTTTTCACCAGCAGGCT-3′) (TAGC, Copenhagen, DK). Primers spanned intron–exon junctions to prevent hybridization to genomic DNA. Product correctness was confirmed by analysis on 2% agarose gel and DNA sequencing (GATC Biotech AG, Germany).



2.5. *Quantification of mRNA with real-time PCR*

Real-time PCR was performed with 10 μL QuantiTect™SYBR®Green PCR mastermix (Qiagen) containing HotStarTaq® DNA polymerase, dNTPs, PCR buffer, and SYBR Green I and 2 μL cDNA as template in a 20 μL reaction volume in the Rotor-Gene 3000™ System (Corbett Research, Australia). Optimization was performed by stepwise variation of annealing temperatures and $MgCl_2$ concentrations. All primers were used at a concentration of 0.5 pmol/μL. The PCR profile was: 95 °C for 15 min, 45 cycles consisting of 95 °C for 15 s, 56–59 °C for 30 s and 72 °C for 30 s. The mRNA expression of each gene was measured in individual runs containing all test samples. Parallel analyses of dilutions of aorta cDNA from intra-run samples were used to determine the relation between the time point of the log–linear increase of the fluorescence signal and the concentration of mRNA transcript. The expression of the target genes was normalized to the expression of HPRT in each sample. Target and HPRT mRNAs were quantified in duplicate. All values were expressed as fractions of the average in the entire run to reduce between-run variation and data are in arbitrary units. The inter-assay coefficients of variation (CV%) were 17% for HPRT, 32% for VG-Ca^{2+}, 13% for BK_{Ca}, 27% for K_{ATP}, 18% for PKG1α and 24% for eNOS mRNA expression.

2.6. *Statistical analysis*

Pre-planned pair wise comparisons of the groups placebo versus E_2, E_2 + MPA or E_2 + NETA; E_2 versus E_2 + MPA or E_2 + NETA; MPA versus NETA or E_2 + MPA versus E_2 + NETA were made by Students *t*-test. One-way analysis of variance (ANOVA) was performed where appropriate. Values are expressed as mean ± S.D. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. *Uterine weights, cholesterol levels and vessel characteristics*

The effect of E_2 treatment was confirmed by an increase in the uterus weight. Lower uterine weights in the groups receiving progestins confirmed the intake of progestins (Table 1). The median serum-E_2 concentration in the group receiv-

Fig. 1 – Vascular reactivity in isolated coronary arteries from hyperlipidemic rabbits. Curves show coronary artery contractile or dilatory response to sodium nitroprusside (SNP) (10^{-9} to 10^{-2} M (A), potassium (K^+) (5.8–50 mM) (B), calcium chloride, Ca^{2+} (10^{-6} to 10^{-2} M) (C) and nimodipine (10^{-10} to 10^{-7} M) (D) after treatment with placebo (open square) or E_2 (closed square) for 16 weeks. Data are expressed as active wall tension (N/m) or % of precontraction induced by 50 mM K^+, symbols with bars indicate mean ± S.E.M., lines show the sigmoidal curve fits, $n = 8–10$. $^*P < 0.05$ vs. placebo (Student's *t*-test) (E_{max}).

Table 2 – E_{max} and EC_{50} determined by myography in coronary arteries from hyperlipidemic rabbits

Treatment group	SNP			K^+			$CaCl_2$			Nimodipine		
	E_{max} (%)	−log [EC_{50}]	n	E_{max} (N/m)	−log[EC_{50}]	n	E_{max} (N/m)	−log[EC_{50}]	n	E_{max} (%)	−log[EC_{50}]	n
Placebo	79.6 (12)	5.59 (0.4)	9	7.29 (3.5)	1.83 (0.2)	8	9.47 (0.4)	3.46 (0.2)	9	82.5 (5)	8.21 (0.3)	10
E_2	102.3 (20)*	5.52 (0.6)	9	4.36 (2.0)*	1.74 (0.2)	9	8.95 (2.0)	3.49 (0.1)	10	80.6 (5)	8.19 (0.4)	10
E_2 + MPA	86.8 (9)*†	4.90 (0.8)*	10	4.31 (2.5)	1.78 (0.2)	9	8.16 (1.9)	3.37 (0.3)	9	75.5 (13)	8.50 (0.2)*†	9
E_2 + NETA	89.6 (29)	5.35 (1.1)	9	4.22 (1.2)*	1.76 (0.1)	10	9.13 (2.0)	3.33 (0.3)	10	77.3 (4)*	8.24 (0.4)	9
MPA	81.5 (12)	5.07 (0.5)	10	2.62 (0.3)	1.72 (0.1)	10	9.25 (1.9)	3.42 (0.2)	10	79.5 (5)	8.31 (0.3)	10
NETA	84.7 (15)	5.30 (0.7)	10	7.00 (2.0)§	1.93 (0.2)§	7	8.50 (2.6)	3.39 (0.3)	9	77.0 (5)	8.33 (0.3)	8
Placebo (hetero)	89.1 (14)	5.61 (0.6)	13	5.62 (2.1)	1.83 (0.2)	9	8.82 (1.8)	3.51 (0.2)	13	78.2 (9)	8.20 (0.2)	11

Values are given as mean (S.D.). Vascular reactivity of isolated coronary artery segments in response to sodium nitroprusside (SNP), potassium (K^+), calcium chloride ($CaCl_2$) and nimodipine after hormone treatment. E_2: 17β-estradiol; MPA: medroxyprogesterone acetate; NETA: norethindrone acetate; hetero: heterozygous WHHL rabbits. The maximum contractile and dilatory responses (E_{max}) are given as N/m and % of precontraction, respectively, −log[EC_{50}] is the negative logarithm of the concentration (mol/L) required to produce half the maximum response. *$P < 0.05$ vs. placebo and †$P < 0.05$ vs. E_2, §$P < 0.05$ vs. MPA (Student's t-test).

ing E_2 alone was 120 pmol/L, this corresponds to serum-E_2 concentrations measured previously under similar experimental conditions [30]. The plasma total cholesterol concentration was significantly higher in the homozygous WHHL rabbits compared to the heterozygous WHHL placebo group (Table 1). There was no significant difference in the plasma total cholesterol concentration between E_2 and the placebo-treated homozygous WHHL rabbits, whereas the addition of MPA and NETA to E_2 treatment significantly increased the plasma cholesterol concentration (Table 1). None of the coronary artery segments had atherosclerotic lesions on histological examination. The mean luminal diameter of the coronary arteries (Table 1) and the maximal contractile response to 124 mM K^+ PSS (data not shown) were similar in all groups.

3.2. *No effect of hyperlipidemia on coronary artery contraction and dilatation*

There was no significant difference in the degree of potassium and calcium-induced contraction or nimodipine and sodium nitroprusside-induced dilatation between the placebo-treated homozygous and heterozygous WHHL rabbits. Also, there was no difference in the magnitude of acetylcholine-induced endothelium-dependent dilatation between the two groups at 10^{-5} M (89.4 ± 6.3 ($n = 5$) versus 86.3 ± 6.1 ($n = 7$), % of precontraction).

3.3. *Effects of E_2 on vascular reactivity in response to SNP, K^+, Ca^{2+} and nimodipine*

E_2 increased the maximum dilatation in response to SNP ($P < 0.05$, Fig. 1A) and reduced the maximum contraction in response to extracellular K^+ in coronary arteries when compared to placebo ($P < 0.05$, Fig. 1B). Vasoconstriction induced by $CaCl_2$ and vasodilatation induced by nimodipine was unaffected by E_2 (Fig. 1C and D) (Table 2).

3.4. *Effects of combining E_2 with MPA or NETA on vascular reactivity in response to SNP, K^+, Ca^{2+} and nimodipine*

The E_2-induced increase in maximum dilatation in response to SNP was abolished when E_2 was combined with MPA but not when E_2 was combined with NETA (E_{max}, $P < 0.05$, Fig. 2A). The E_2-induced reduction in vasocontraction in response to extracellular K^+ was maintained when E_2 was combined with either MPA or NETA (Fig. 2B). Also, NETA but not MPA in combination with E_2 significantly reduced the potassium-induced maximum contraction when compared to placebo (E_{max}, $P < 0.05$, Table 2). The addition of MPA or NETA to E_2 had no effect on $CaCl_2$-induced vasoconstriction compared to E_2 alone (Fig. 2C). However, addition of MPA but not NETA treatment to E_2 significantly enhanced the dilatation after nimodipine stimulation as compared to E_2 alone (log EC_{50}, $P < 0.05$, Fig. 2D). K^+-induced contraction was reduced by MPA alone as compared to NETA alone, otherwise there was no significant difference in E_{max} or EC_{50} between MPA and NETA alone or between E_2 + MPA and E_2 + NETA in response to any of the vasoactive substances (Table 2).

3.5. *Effect of E_2 alone and in combination with MPA or NETA on BK_{Ca}, β, $K_{ATP,Kir\,6.1}$, PKG1α, L-type VG-Ca^{2+} and eNOS mRNA expression*

The BK_{Ca}, K_{ATP}, PKG1α and eNOS mRNA expression in coronary arteries was unaffected by hormone treatment (Fig. 3A–C and data not shown). E_2 in combination with MPA increased the expression of L-type VG-Ca^{2+} channel mRNA compared to E_2 (1.48 ± 0.45 ($n = 8$) versus 1.12 ± 0.24 ($n = 9$)), arbitrary units, $P < 0.05$, Fig. 3D). However, E_2 alone or in combination with NETA had no effect on L-type VG-Ca^{2+} channel mRNA compared to placebo (Fig. 3D).

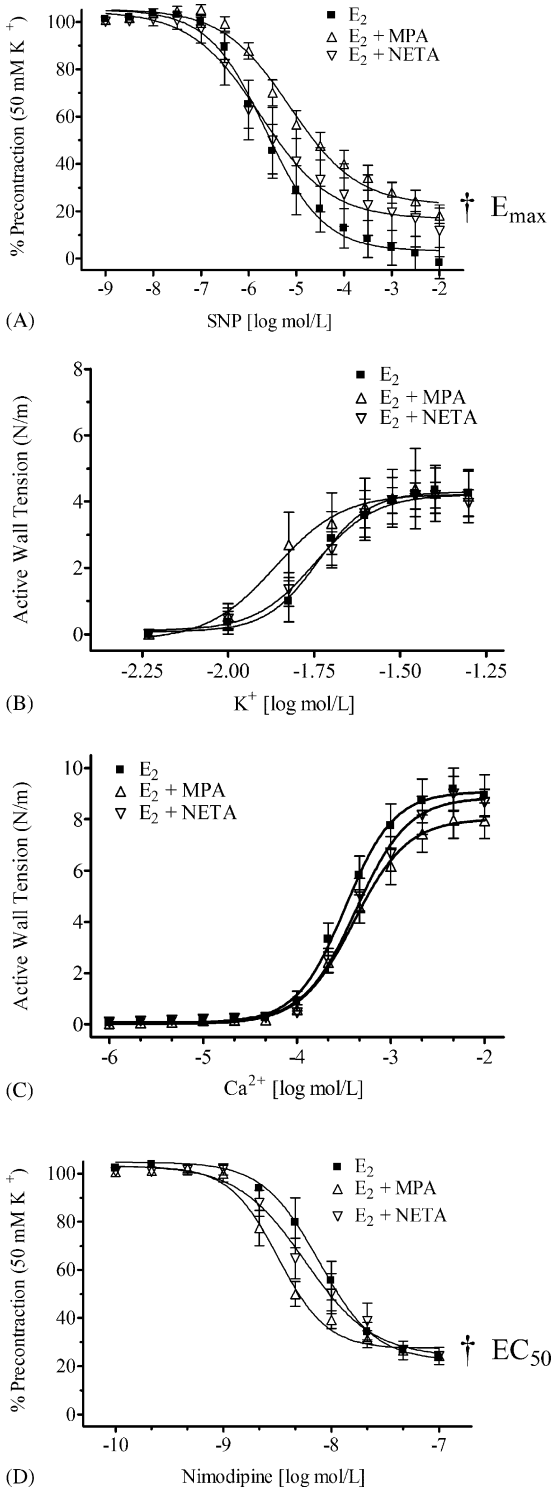


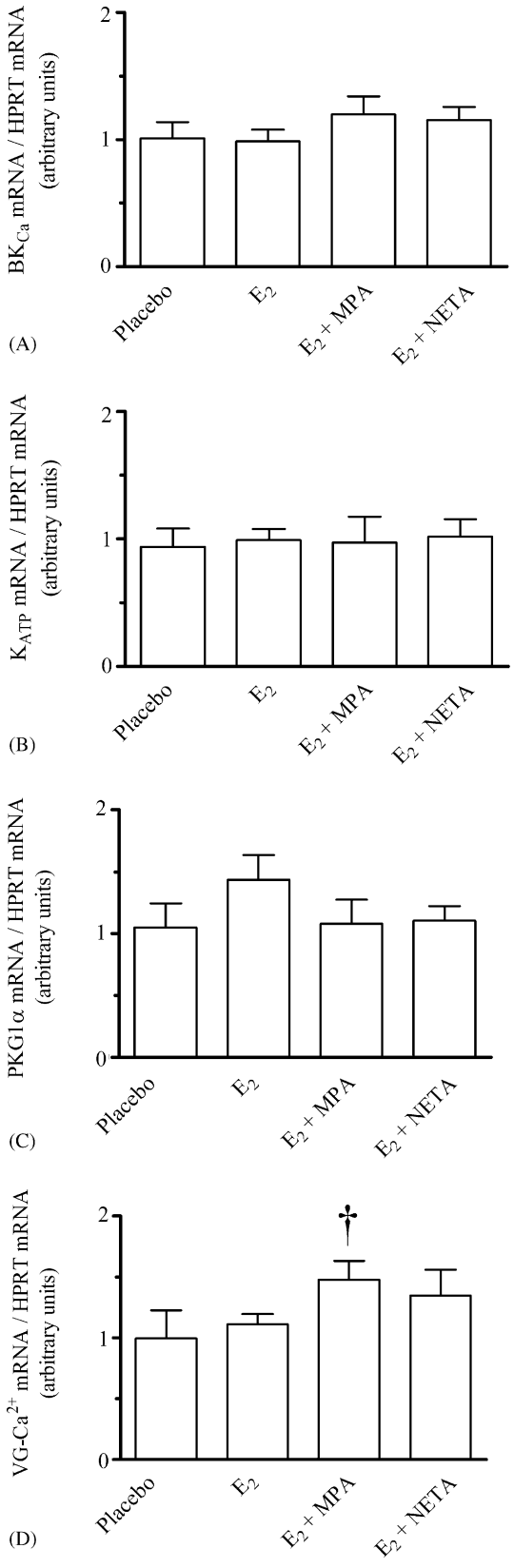
Fig. 2 – Vascular reactivity in isolated coronary arteries from hyperlipidemic rabbits. Curves show coronary artery contractile or dilatory response to sodium nitroprusside (SNP) (10^{-9} to 10^{-2} M) (A), potassium (K^+) (5.8–50 mM) (B), calcium chloride, Ca^{2+} (10^{-6} to 10^{-2} M) (C) and nimodipine (10^{-10} to 10^{-7} M) (D) obtained after treatment with E_2 (closed square), E_2+MPA (triangles), or E_2 + NETA (inverted triangles) for 16 weeks. Contractile responses are expressed as active vessel wall tension (N/m) and dilatory responses are expressed as % of precontraction induced by 50 mM K^+, symbols with bars indicate mean ± S.E.M., lines show the sigmoidal curve fits, $n = 8$–10. †$P < 0.05$, E_2 vs. E_2+MPA (Student's *t*-test) (E_{max} or EC_{50}).

4. Discussion

The present study examined the effects of long-term treatment with E_2 alone or in combination with MPA or NETA on the vasomotor response and gene expression of vascular ion channels. The results confirm a beneficial vascular effect of long-term E_2 treatment and suggest that MPA may modulate the effect of E_2 in isolated coronary arteries from hyperlipidemic rabbits.

In accordance with previous observations, our study showed that long-term E_2 treatment decreases K^+-induced contraction [31,32]. The attenuated contractile response to membrane depolarization after K^+-stimulation may reflect increased K^+ channel activity and/or decreased VG-Ca^{2+} channel activity. Membrane depolarization after high extracellular K^+ stimulation may reduce the influence of K^+ channels, since depolarization primarily causes contraction by increasing Ca^{2+}-influx through VG-Ca^{2+} channels [33], thus, it is possible that attenuation of VG-Ca^{2+} channel activity rather than K^+ channel activity explains the observed effect of E_2 on K^+-induced vasocontraction. The possible involvement of VG-Ca^{2+} in the long-term effect of E_2 is supported by the observed increase in vasodilatation after stimulation with an NO-donor, i.e. SNP, since NO causes relaxation through direct effects on VG-Ca^{2+} rather than through activation of K^+ channels [21] in vessels precontracted with high K^+ (50 mM). In addition the lack of K^+ channel involvement is supported by the finding that E_2 had no regulatory effect on BK_{Ca} or K_{ATP} mRNA expression. The findings are thus indicative of K^+ channel independent effects of E_2 treatment. However, the possible involvement of VG-Ca^{2+} in the effect of E_2 treatment was not supported by the present results since E_2 had no effect on vasoconstriction induced by extracellular $CaCl_2$ or vasodilatation induced by L-type VG-Ca^{2+} blockade with nimodipine. Furthermore, this study found no regulatory effect of E_2 treatment on L-type VG-Ca^{2+} channel mRNA expression, suggesting that posttranscriptional activation or transcription of other genes is causing the observed effects of E_2 treatment on vasomotion. The lack of an effect of long-term E_2 treatment on $CaCl_2$-induced vasocontraction is in accordance with previous findings [32], although it has recently been suggested that E_2 reduces mRNA

expression of the VG-Ca^{2+} α_{1C} subunit in the rat aorta [34]. Of note, in the present study the mechanisms involved in the potentially beneficial effects of long-term E_2 treatment on vascular function, could not be explained by gene regulation of the β-subunit of the BK_{Ca}, the Kir6 pore-forming subunit of the K_{ATP}, PKG1α or the L-type VG-Ca^{2+} channel. The lack of a difference in these genes could possibly be due to the rather high CV% which may have introduced a Type II error.



Interestingly, MPA modulated the effect of E_2 on SNP- and nimodipine-induced vasodilatation. The modulatory effect of MPA on nimodipine-induced dilatation was confirmed by an increase in L-type VG-Ca^{2+} mRNA expression. This suggests that although long-term E_2 treatment alone had no detectable effect on nimodipine-induced dilatation or L-type VG-Ca^{2+} mRNA expression, MPA in combination with E_2 may exert a potentially detrimental effect on vascular function through this ion channel. An increase in the potency of nimodipine-induced dilatation, suggests that the number of and/or open probability of VG-Ca^{2+} channels that were initially stimulated by extracellular K^+-induced depolarization were increased. The augmentation of VG-Ca^{2+} channel function by MPA treatment is also supported by the finding that MPA opposed vasodilatation induced by direct NO donor stimulation under membrane depolarized conditions. The results show a modulatory effect of long-term MPA treatment on calcium mediated mechanisms in coronary arteries and are in accordance with previous findings demonstrating that MPA in combination with E_2 interferes with calcium mediated mechanisms in isolated smooth muscle cells [35]. The abrogating effect of MPA on vascular function is consistent with previous observations in postmenopausal women [36–39] although other studies have reported no adverse effects of concomitant administration of MPA [40–43].

In contrast to MPA, NETA did not significantly attenuate the effects of E_2 treatment on SNP- and nimodipine-induced vasodilatation. Of note, although MPA treatment alone reduced K^+-induced contraction compared to NETA alone, overall, the proposed differential effect of MPA and NETA in combination with E_2 when compared to E_2 alone where not reflected in differential effects between the two combination treatments.

The biological effect of NETA is dependent on its conversion to the active progestational metabolite norethindrone (NET) and importantly the conversion of NETA to NET occurs in rabbits [44]. In contrast to MPA, NETA and its metabolites have estrogenic effects [45–48]. Thus, the estrogenic effects of NETA may help explain the difference in the vascular actions of NETA and MPA. However, variability in the androgenic, mineralocorticoid and glucocorticoid actions of progestins [49] may also help explain the present findings and previous observations of different effects of different progestins on smooth muscle proliferation and migration, connective tissue synthesis, generation of reactive oxygen species, carbohydrate metabolism, inflammation and coagulation/fibrinolysis may also influence vascular function [3,50].

Fig. 3 – Expression of mRNAs in isolated coronary arteries from hyperlipidemic rabbits. Column bars show the expression of BK_{Ca}, β subunit (A), $K_{ATP,Kir\,6.1}$ (B), PKG1α (C) and VG-Ca^{2+} (D) mRNA after treatment with placebo, E_2 alone or combined with either MPA or NETA for 16 weeks. Data are normalized to HPRT and expressed in arbitrary units, vertical bars indicate mean ± S.E.M., $n = 8–10$. $^{\dagger}P < 0.05$ vs. E_2 (Student's *t*-test).

In conclusion, the present data suggest that when E_2 is administered with MPA, effects of E_2 on nitric oxide and Ca^{2+}-mediated vascular reactivity in rabbit coronary arteries are modulated. Although, clearly further investigations are needed to understand how progestins interact with estrogen during long-term treatment the results suggest that the progestin component in HRT may interfere with the vascular effects of estrogen.

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