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RESEARCH PAPER

Differential effects of medroxyprogesterone acetate on thrombosis and atherosclerosis in mice

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Background and purpose: The risk for cardiovascular events including venous and arterial disease and stroke is elevated after treatment with estrogen and medroxyprogesterone acetate (MPA) in postmenopausal women. Here, we have investigated the effect of MPA on arterial thrombosis and atherosclerosis in a murine model of atherosclerosis.
Experimental approach: Apolipoprotein E (ApoE)$^{-/-}$ mice were bilaterally ovariectomized and treated with placebo, MPA (27.7 μg·day^{-1}) and MPA + 17-β-oestradiol (E_2; 1.1 μg·day^{-1}) for 90 days, on a Western-type diet. Thrombotic response was measured in a photothrombosis model, platelet activation by fluorescence activated cell sorting (FACS) analysis (CD62P) and thrombin generation by the endogenous thrombin potential (ETP). Furthermore, aortic plaque burden and aortic root plaque composition were determined.
Key results: MPA and MPA + E_2-treated animals showed an aggravated thrombotic response shown by significantly reduced time to stable occlusion. The pro-thrombotic effect of MPA was paralleled by increased ETP whereas platelet activation was not affected. Furthermore, MPA + E_2 reduced the number of cells positive for α-smooth muscle actin and increased hyaluronan in the plaque matrix. Interestingly, total plaque burden was reduced by MPA but unchanged by MPA + E_2.
Conclusion and implications: Long-term treatment with MPA and MPA + E_2 increased arterial thrombosis despite inhibitory effects of MPA on atherosclerosis in ApoE-deficient mice. Increased thrombin formation, reduced smooth muscle content and remodelling of non-collagenous plaque matrix may be involved in the pro-thrombotic effects. Thus, MPA exhibits differential effects on arterial thrombosis and on atherosclerosis.
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Abbreviations: BSA, bovine serum albumin; CEE, conjugated equine estrogens; CHD, coronary heart disease; E_2, 17-β-oestradiol; ECM, extracelluar matrix; ETP, endogenous thrombin potential; FITC, fluorescein-isothiocyanate; HA, hyaluronan; HAbP, hyaluronan binding protein; HRT, hormone replacement therapy; MPA, medroxyprogesterone acetate; OVX, ovariectomized; PBS, phosphate-buffered saline; PE, phycoerythrin; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SMC, smooth muscle cells; SNAP, S-nitroso-N-acetyl-D, L-penicillamine; WHI, World Health Initiative

Introduction

Hormone replacement therapy (HRT) was expected to prevent cardiovascular events in postmenopausal women. This assumption was based on several epidemiological and observational studies suggesting that postmenopausal oestrogen therapy would reduce mortality from coronary heart disease (CHD) (Stampfer *et al.*, 1985; Grodstein *et al.*, 2000). Furthermore, animal studies in monkeys and mice also suggested anti-atherosclerotic effects of oestrogen substitution (Adams *et al.*, 1990; Bourassa *et al.*, 1996). However, recently large prospective randomized clinical trials questioned this concept and revealed increased thromboembolic event rates, including stroke (Rossouw *et al.*, 2002; Manson *et al.*, 2003; Wassertheil-Smoller *et al.*, 2003; Cushman *et al.*, 2004). Therefore, the concept that oestrogens are generally protective against CHD in women was abandoned and long-term HRT is no longer recommended (Rossouw *et al.*, 2002). However, it is considered that oestrogens might protect from atherosclerosis

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only in young women if initiated early after menopause, whereas treatment of women with advanced atherosclerosis, years or decades after menopause might lead to worsening of clinical outcomes ('window of opportunity') (Dubey *et al.*, 2005; Rossouw *et al.*, 2007).

A trend towards inhibition of CHD was observed in the arm of the Women's Health Initiative (WHI) trial with conjugated equine oestrogens (CEEs) alone (Anderson *et al.*, 2004). In contrast in the arm receiving CEE plus medroxyprogesterone acetate (MPA) a trend towards increased CHD was recorded, which might be an indication of an effect of MPA on cardiovascular disease and perhaps atherothrombosis (Rossouw *et al.*, 2002). Comprehending the role of progestin therapy is, however, complicated by the fact that many progestin derivatives have been developed that differ with respect to the concomitant activation or antagonism of other steroid receptors. MPA activates glucocorticoid receptors, which could mimic part of the anti-atherosclerotic effects of glucocorticoids (Asai *et al.*, 1993), and has, on the other hand, also anti-androgenic activity, which might diminish protective oestrogen effects. In monkeys, MPA interferes with anti-atherosclerotic oestrogen effects whereas progesterone does not (Adams *et al.*, 1990; Adams *et al.*, 1997). Furthermore, the effect on endothelial cells is variable between the progestins; for instance, progesterone increases endothelial nitric oxide (NO) release whereas MPA does not, a finding attributed to differences in the downstream signalling in endothelial cells (Simoncini *et al.*, 2004).

MPA is currently used in addition to HRT also in premenopausal women as a contraceptive and to treat various gynaecological conditions such as endometriosis, polycystic ovarian syndrome and irregular uterine bleeding (Cullins, 1996). Further research on the effects of MPA on atherothrombosis in animal models will contribute to a better understanding of its effects on CHD and the underlying mechanisms of this frequently used progestin. Therefore, the aim of the present study was to evaluate the effect of MPA alone or in combination with oestradiol on arterial thrombosis and atherosclerosis in apolipoprotein E (ApoE)-deficient mice.

Methods

Animals

All animal care and experimental procedures complied with the guidelines for the use of experimental animals as outlined in the 'Deutsches Tierschutzgesetz' and according to the 'Guide for the Care and Use of Laboratory Animals' (NIH publication 85–23, revised 1985). Homozygous ApoE-deficient mice (strain: Maeda) were obtained from Taconic M&B (Ejby, Denmark). Mice were kept in a 12 h light/dark cycle with access to food and water *ad libitum*. At weaning (age 28 days) mice were bilaterally ovariectomized (OVX). Anaesthesia was performed using a mixture of ketamine (100 $mg \cdot kg^{-1}$) and xylazine (5 $mg \cdot kg^{-1}$). Mice were randomly assigned to three treatment groups, namely, placebo, MPA and MPA + 17-β-oestradiol (E_2). At 42 days of age, placebo pellets or slow-release hormone pellets (Innovative Research of America), prepared to dispense 27.7 $\mu g \cdot day^{-1}$ MPA or 1.1 $\mu g \cdot day^{-1}$ E_2 for 90 days, were implanted subcutaneously. The dose of oestrogen used in our study was based on earlier studies by Elhage *et al.* (1997) who observed maximal inhibition of fatty streak formation with E_2 at doses between 0.83 $\mu g \cdot day^{-1}$ and 1.6 $\mu g \cdot day^{-1}$. The dose of MPA used in our study was based on earlier studies by Shultz *et al.* (2004) and Hanke *et al.* (1996) who used progestin doses equalling a progestin/oestrogen ratio between 10:1 and 100:1. The treatment period was based on earlier studies performed by Bourassa *et al.*, (1996) and Marsh *et al.* (1999) who described anti-atherosclerotic actions of E_2. Starting at the day of pellet-implantation mice were fed a Western-type diet containing 21% butter fat and 0.15% cholesterol by weight (Ssniff, Soest, Germany). Animal weights were determined after 132 days. The experimental design is summarized in Figure 1A. The drug/molecular target nomenclature used here follows Alexander *et al.* (2008).

Determination of aortic plaque burden

Animals were perfusion fixed with 4% paraformaldehyde, aortas were carefully removed and postfixed with 4% paraformaldehyde (4°C, overnight). The next day aortas were transferred into 1× phosphate-buffered saline (PBS), freed from adventitia and subsequently equilibrated in 78% methanol for 5 min. Subsequently, Oil-Red-O staining was performed for 90 min at room temperature and aortas washed in 78% methanol for 10 min afterwards. Finally *en face* pictures were taken and the percentage of Oil-Red-O stained area of total surface area was determined by ImageJ 1.37v software (National Institutes of Health, Bethesda MD, USA) and defined as plaque burden. Eighteen to 21 mice were analysed in each treatment group.

Measurement of lipoprotein profiles

Total cholesterol and triglycerides were determined after feeding by the department of Clinical Chemistry of the University Clinics Düsseldorf according to standard procedures (enzymatic colour-reaction kits; Chol and TG, cobas, Roche, Mannheim, Germany). Ten to 13 mice were analysed in each treatment group.

Photochemical induction of thrombosis

Thrombosis of the right carotid artery was induced by injection of Rose Bengal and subsequent irradiation using a green light laser as previously described (Wilson *et al.*, 2003). Time to first occlusion was defined as the time when blood-flow first ceased to zero. Time to stable occlusion was defined as the time at which blood-flow stayed at zero for ≥10 min. The frequency of flow recovery was determined in the interval between first and stable occlusion. Every time when the blood-flow increased to more than 0.09 mL/min after an intermediate occlusion was defined as one event of flow-recovery. The sum of these events was set as frequency of flow recovery. Two out of 10 placebo mice and one out of nine MPA + E_2 animals died before stable occlusion and were excluded. Finally, 6–7 mice were analysed in each treatment group.

Analysis of platelet activation and endogenous thrombin potential

Whole blood was obtained by cardiac puncture of mice anaesthetized with a mixture of ketamine (100 $mg \cdot kg^{-1}$) and

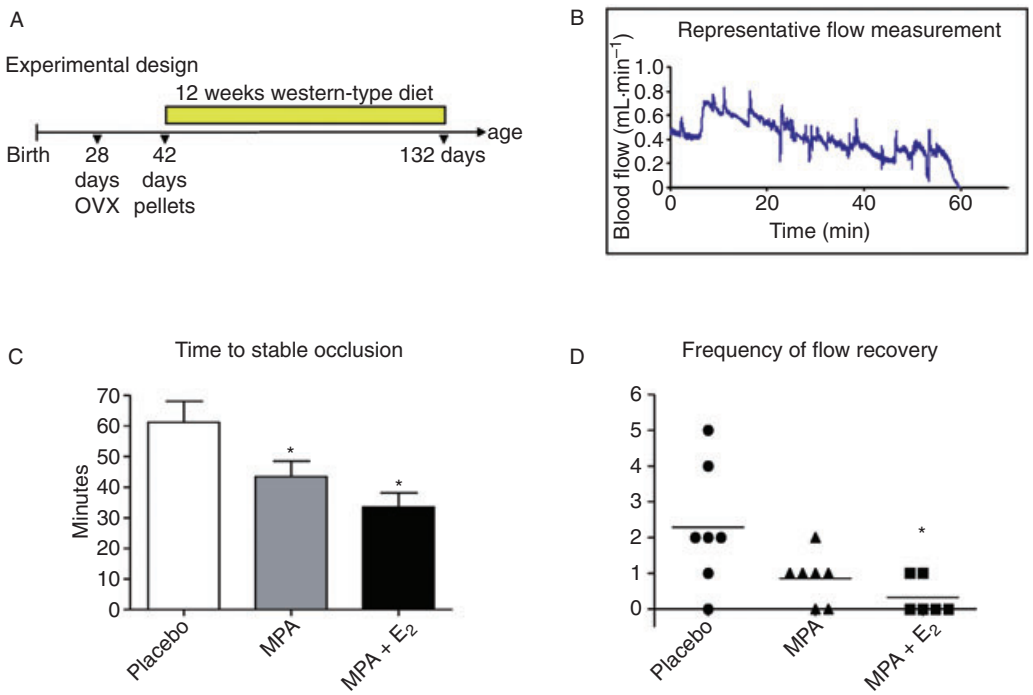

Figure 1 Thrombotic response. (A) Experimental design. (B) Representative blood-flow measurement after initiation of photochemical injury at time 0. (C) Times to stable occlusion from mice receiving placebo, medroxyprogesterone acetate (MPA; 27.7 μg·day^{-1}) and MPA + E_2 (1.1 μg·day^{-1}). (D) Graph showing the frequency of flow-recovery as a measure of thrombus stability in mice treated with placebo, MPA and MPA + E_2. Data represent means ± standard error of the mean; n = 6–8, *, $P < 0.05$ versus placebo.

xylazine (5 mg·kg^{-1}). Sodium citrate (0.02 M, final concentration) was used as anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation at 850× g for 45 s and diluted 1:10 with PBS. The percentage of platelets in PRP was determined after incubation of 25 μL PRP with 5 μL phycoerythrin (PE)-conjugated rat anti-mouse anti-CD41 antibody (BD Pharmingen, Heidelberg, Germany) for 30 min and diluted with 500 μL isotone for analysis on a Cytomics FC 500 Cytometer (Beckman Coulter, Krefeld, Germany). PE-conjugated mouse IgG1-PE (Beckman Coulter) was used as isotypic control. PRP was characterized by 98–99% CD41 positive particles. Platelet P-selectin (CD62P) expression as read out for platelet activation was determined by FACS analysis using CD62P-antibody (FITC-conjugated rat-anti-mouse anti-CD62P antibody, BD Pharmingen) and the respective FITC-conjugated isotypic control (mouse IgG1-FITC, Beckman Coulter) as previously described (Zimmermann *et al.*, 2003). To detect differences in the magnitude of maximal platelet activation, PRP was preincubated with convulxin (5 μg/mL, Alexis, Lörrach, Germany) for 5 min and subsequently CD62P surface expression was detected as described above. Analysis was performed using CXP Analysis Software 2.2. Platelet-poor plasma (PPP) was prepared from the PRP and used for the measurement of endogenous thrombin potential (ETP) using a modified thrombinoscope method (Stampfuss *et al.*, 2005). Briefly, 15 μL PPP, 10 μL platelet membranes, 1 pmol innovin as tissue factor source (final concentration), 55 μL PBS and 20 μL recalcification buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.35, 60 mg/mL bovine serum albumin (BSA) and 100 mM Ca^{2+} (final concentration)] containing the fluorogenic substrate (Z-Gly-Gly-Arg-7-amino-4-methylcoumarin; Bachem, Weil am Rhein, Germany) were mixed and thrombin generation was monitored for 60 min using a Fluoroscan Ascent plate reader (Fluoroskan Ascent, Thermo Electron Corporation, Vantaa, Finland). Resulting curves were analysed using Thrombinoscope Analysis 3.0 software. For these mechanistic studies on platelet activation and thrombin generation in response to hormone treatment a separate group of OVX mice was treated as described earlier but for only 2 weeks.

Contraction-relaxation experiments

Endothelial function was examined in 5 mm thoracic aortic ring segments as previously described (Suvorava *et al.*, 2005). Briefly, mice were killed by CO_2, aortas were cautiously removed and aortic rings prepared. Subsequently aortic rings were repeatedly depolarised with 80 mM KCl. Aortic rings were precontracted submaximally with 200 nM phenylephrine and endothelium-dependent relaxation induced by cumulative addition of acetylcholine (10^{-9}–10^{-5} M). Endothelium-independent relaxation to increasing concentrations of the NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (10^{-10}–10^{-5} M) was examined after precontraction with increasing concentrations of phenylephrine (10^{-9}–10^{-5} M). Two aortic rings from each animal were measured and the resulting data averaged. Five to seven mice were analysed in each treatment group.

Histochemistry and immunohistochemistry

Animals were perfusion fixed with 4% paraformaldehyde, tissues were excised and post-fixed with 4% paraformaldehyde

(4 h) and transferred to 20% sucrose (overnight) before embedding at –40°C in Tissue Tek® medium according to routine procedures. Aortic root sections (14 μm) were fixed in ice-cold acetone for smooth muscle α-actin (α-SM-actin staining), in 96% ethanol for hyaluronic acid (HA) and Mac2 double staining or in 10% formalin for Oil-Red-O staining, for 15 min at 4°C. Primary antibody against α-SM-actin, (1:50; Abcam, Cambridge, UK) was used and α-SM-actin was detected by a sheep anti-rabbit Cy3 conjugate (1:200, Sigma, Steinheim, Germany). The accumulation of HA and the retention of macrophages in aortic root plaques was analysed by HA/Mac2 double-staining. Slides were incubated with biotinylated hyaluronic acid-binding protein (HAbP; 2 $\mu g \cdot mL^{-1}$; Seikagaku, Tokyo, Japan) at 4°C overnight. After three washes with PBS sections were incubated with streptavidin-FITC (1:200, Dako, Glostrup, Denmark). Subsequently, retention of macrophages was analysed by staining macrophage-antigen Mac2 with a rat-anti-mouse Mac2 first antibody and a rhodamineX-coupled goat-anti-rat IgG as secondary antibody on the same sections. Nuclear staining was performed by using ProLong® Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen, Eugene, OR, USA). The extent of lipid deposition in aortic root plaques was evaluated by Oil-Red-O staining. 5–9 mice were analysed in each treatment group.

Image analysis

Images of all stained samples were captured at 40x magnification using a BX-50 microscope (Olympus, Hamburg, Germany) and ColorView II camera (Soft Imaging System) and AnalySIS 3.2 software (Soft Imaging System, Münster, Germany). For the quantification of α-SM actin, macrophages, hyaluronan and lipid deposits, ImageJ 1.37v software (National Institutes of Health) was used. A colour deconvolution tool was applied to 8 bit converted images to filter out the positively stained areas. Choice of threshold values and quantification of threshold-marked areas were performed as previously described (Dai *et al*., 2007). Areas of plaque deposition in the aortic root were identified morphologically and measurements were only performed there. Measurements from at least five sections were averaged.

Statistical analysis

Data are presented as mean ± standard error of the mean of the number of (*n*) mice. Statistical significance among the concentration-response curves was analysed by two-way analysis of variance and one-way analysis of variance was used for plaque score, quantitative image analysis, thrombosis, CD62P-expression analysis and ETPs. Frequency of flow recovery was analysed using the Kruskal–Wallis test. *P* values < 0.05 were considered as statistically significant.

Results

Arterial thrombosis

The experimental design is depicted in Figure 1A. OVX caused significant uterine atrophy, demonstrating that the procedure was effective (data not shown). Total plasma cholesterol and triglyceride levels (Table 1) were not affected by either form of hormone treatment. However, the combined treatment with MPA and E_2 significantly decreased body weight of mice compared with placebo, whereas animals treated with MPA alone showed no effects on body weight (Table 1). After photochemical induction of thrombus formation, the time to stable occlusion of the right carotid artery was determined. Figure 1B shows a representative chart of the decrease of blood-flow after photochemical injury. MPA significantly shortened times to occlusion of the right carotid artery (Figure 1C) suggesting an aggravated thrombotic response. In addition MPA caused a trend towards reduced frequency of flow-recovery (Figure 1D) as compared with placebo-treated animals suggesting increased stability of newly formed platelet aggregates or increased adhesion of the thrombus to the vessel wall. Both parameters, time to occlusion and frequency of flow recovery were also affected by MPA + E_2 (Figure 1C,D).

Table 1 Body weight, total cholesterol and triglycerides

	Body weights (g)	*Total cholesterol ($mg \cdot mL^{-1}$)*	*Triglycerides ($mg \cdot mL^{-1}$)*
Placebo	35.0 ± 0.5	8.1 ± 0.8	0.9 ± 0.1
MPA	34.2 ± 0.6	8.9 ± 0.8	1.1 ± 0.1
MPA + E_2	28.7 ± 0.9*	7.1 ± 0.7	1.0 ± 0.2

Body weight and lipid parameters after feeding were determined at the end of the experimental period at the age of 132 days, *$P < 0.05$ versus placebo and medroxyprogesterone acetate (MPA).

Platelet activity and endogenous thrombin potential (ETP)

To address the mechanisms that might be responsible for the pro-thrombotic effect of MPA and MPA + E_2, platelet activation and thrombin generation were determined. FACS analysis of CD62P revealed no differences in the basal expression levels on platelets derived from placebo versus MPA and MPA + E_2 (Figure 2A,B). Activation of platelets with the snake venom convulxin resulted in a four-fold increase of CD62P expression and was not affected by MPA. In the group receiving MPA + E_2, the platelet activation in response to convulxin was even reduced. Taken together the results do not support increased platelet activation as the reason for the shortened time to occlusion in the photothrombosis model. However, thrombin generation was affected by the hormone treatment. MPA treatment caused a significant increase of the ETP that reflects the area under the curve of the thrombin generation time course. In contrast MPA + E_2 did not alter the ETP (Figure 2C,D). Taken together, these experiments suggest that increased thrombin formation as detected by the ETP measurements may explain partially the aggravated thrombotic response in the photothrombosis model after MPA treatment.

Aortic plaque burden

Next, the extent of plaque burden was determined in order to search for the mechanisms that might underlie the increased thrombotic response in atherosclerotic mice chronically treated with MPA. Of note, MPA decreased atherosclerotic plaque burden in the thoracic and abdominal aorta by about 20% (Figure 3A,B). In mice receiving MPA + E_2 no effect

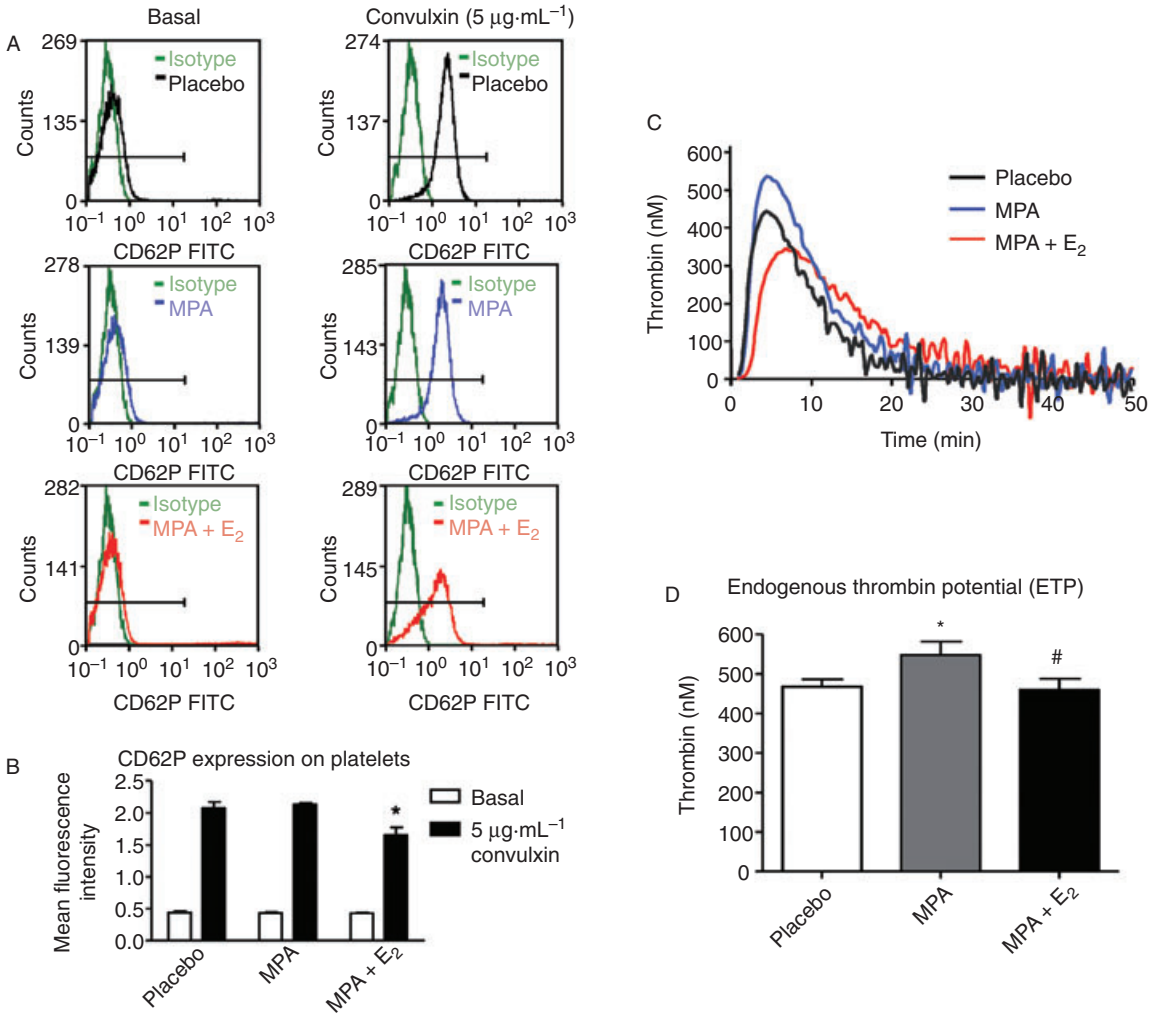

Figure 2 Platelet activation and endogenous thrombin potential (ETP) (A) Representative plots of *ex vivo* FACS analysis of CD62P-expression on platelets in platelet-rich plasma from mice treated with placebo, medroxyprogesterone acetate (MPA; 27.7 μg·day^{-1}ay) and MPA + E_2 (1.1 μg·day^{-1}). Left panel: basal CD62P expression; right panel: CD62P expression after stimulation with 5 μg/mL convulxin (B) Quantitative analysis of CD62P expression. (C) Representative curves for thrombin generation over time (ETP) in mice treated with placebo, MPA and MPA + E_2. (D) Quantitative analysis of ETP. Data represent means ± standard error of the mean; $n = 6–7$, $^*P < 0.05$ versus placebo, $\#P < 0.05$ versus MPA.

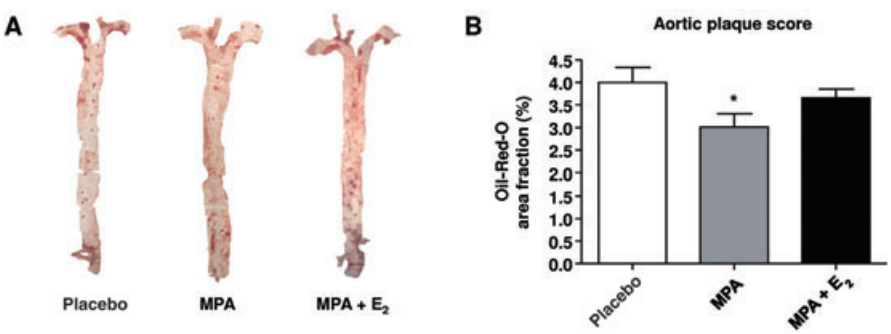

Figure 3 Plaque burden. (A) Oil-Red-O stained aortas from mice substituted with medroxyprogesterone acetate (MPA; 27.7 μg·day^{-1}) and MPA + E_2 (1.1 μg·day^{-1}). (B) Aortic plaque scores from mice treated with placebo, MPA and MPA + E_2. Data represent means ± standard error of the mean; $n = 18–21$; $^*P < 0.05$ versus placebo.

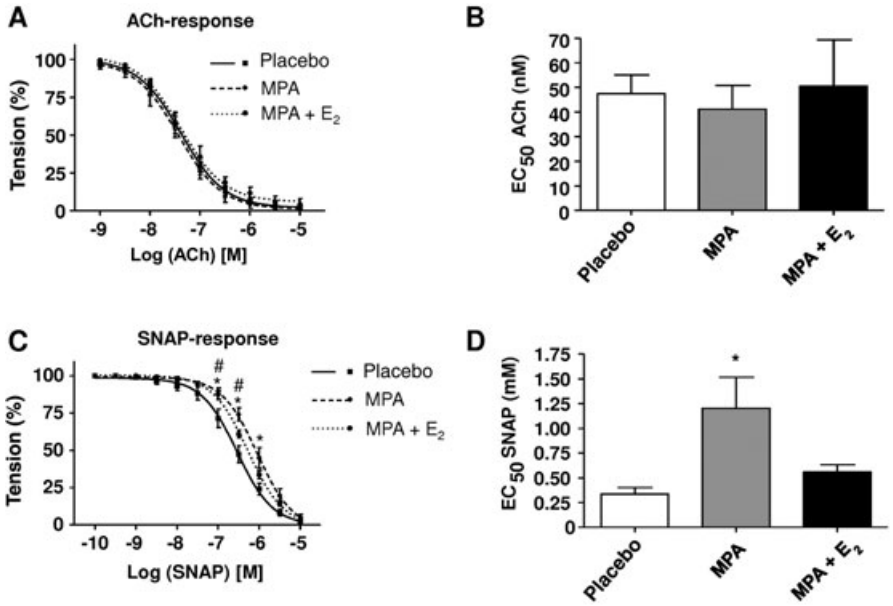

Figure 4 ACh-dependent vasorelaxation of aortic rings. (A) Concentration-response curves in response to ACh of aortic rings derived from mice treated with placebo, medroxyprogesterone acetate (MPA; 27.7 μg·day^{-1}) and MPA + E_2 (1.1 μg·day^{-1}). (B) Half maximal effective concentration (EC_{50}) values for ACh-induced vasorelaxation. (C) Concentration-response curves in response to S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in response to treatment with placebo, MPA and MPA + E_2. (D) EC_{50} values for SNAP. EC_{50} values for ACh calculated from concentration-response curves in (A). EC_{50} values for SNAP calculated from concentration-response curves in (C). Data represent means ± standard error of the mean; n = 5–7, *P < 0.05 for placebo versus MPA, #P < 0.05 for placebo versus MPA + E_2.

on atherosclerosis compared with placebo was detected (Figure 3A,B). Therefore, effects on atherogenesis are unlikely to be responsible for the aggravated thrombogenic response to MPA treatment.

Endothelium-dependent vasorelaxation

Endothelial function was considered as an underlying mechanism affecting both thrombotic responses and atherosclerosis in MPA treated mice. Thus, endothelium-dependent ACh-induced vasorelaxation of thoracic aortic rings of the chronically treated animals was determined in organ bath experiments and found to be unchanged in rings derived from both treatment groups, MPA or MPA + E_2 (Figure 4A,B), excluding the possibility that effects on endothelial function are involved in the pro-thrombotic or anti-atherosclerotic actions of MPA in this model. To evaluate the endothelium-independent effects of MPA and MPA + E_2 on vasorelaxation the response to the exogenous NO-donor SNAP was analysed. Remarkably, thoracic aortic rings from MPA-treated animals showed a significantly impaired relaxation in response to SNAP whereas aortic rings from mice receiving combined treatment did not differ from placebo (Half maximal effective concentration values SNAP: placebo, 0.34 ± 0.07 mM; MPA, 1.20 ± 0.31 mM; MPA + E_2: 0.56 ± 0.08 mM, n = 6–8, Figure 4C,D).

Plaque composition at the aortic root

In addition the composition of atherosclerotic plaques with respect to extracellular matrix (ECM), smooth muscle cells (SMCs) and macrophages was measured. Differentiated SMC in plaques at the aortic root were detected by α-SM-actin staining. Interestingly, MPA caused a trend to reduced α-SM-actin staining that became significant after MPA + E_2 treatment (Figure 5A–C,G). In contrast macrophage content of plaques, as determined by Mac2 immunostaining, was not affected (Figure 5D–F,H). Of note, HA content was significantly elevated after MPA + E_2 (Figure 5D–F,I). Lipid deposits in aortic root plaques (Figure 6A–D), collagen content and collagen fibril packing as evidenced by picro-sirius red staining and birefringence analysis were not affected (not shown). These data on plaque composition clearly indicated remodelling of the non collagenous ECM, namely, HA, and decreased amounts of differentiated SMC in response to MPA and MPA + E_2.

Discussion

The main findings of the present work were that, on the one hand, chronic treatment of $ApoE^{-/-}$ mice with MPA aggravated the response in an experimental model of arterial thrombosis and, on the other hand, slightly reduced plaque burden. The model of photochemically induced thrombosis was chosen, because it is regarded as the most sensitive *in vivo* thrombosis model and one that will detect even subtle changes in the thrombotic response, with the highest sensitivity and accuracy (Westrick *et al*., 2007). In this model, oxidative damage to the endothelium is caused by photochemical activation of Rose Bengal and subsequent adhesion of platelets to the subendothelial matrix and initiation of coagulation (Westrick *et al*., 2007). Both MPA and MPA + E_2 enhanced the thrombotic response compared with the placebo-treated animals. In

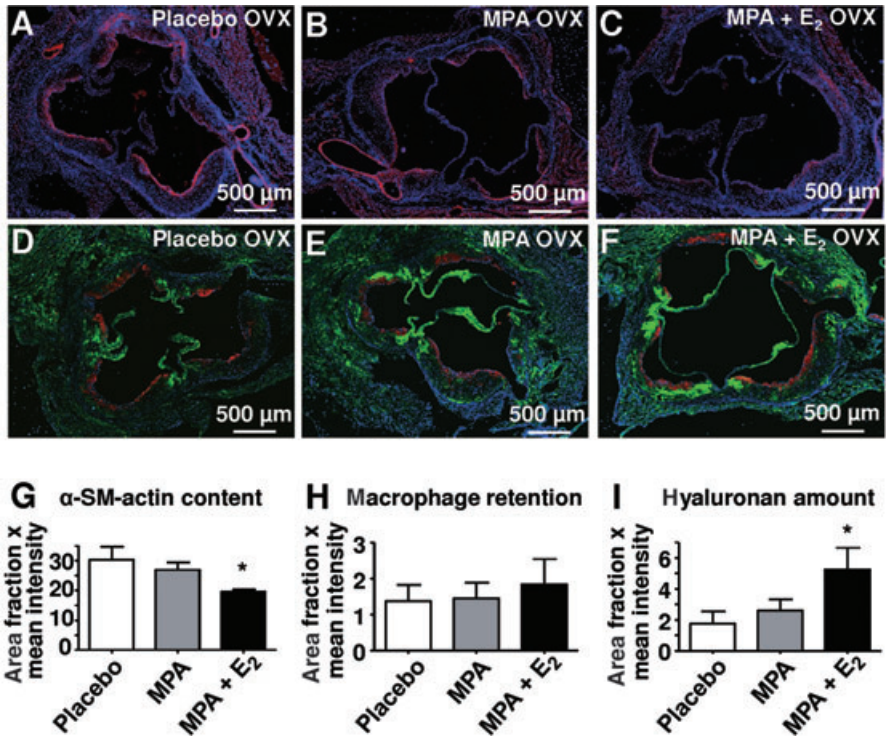

Figure 5 Plaque composition. (A–C, G) Aortic root sections stained for α-SM-actin (red) from mice treated with placebo, medroxyprogesterone acetate (MPA; 27.7 µg·day^{-1}) and MPA + E_2 (1.1 µg·day^{-1}). (D–F, H–I). Aortic root sections stained for hyaluronan and the macrophage antigen Mac2. (G–I) Data represent area fractions of positively stained antigens as determined by digital image analysis; means ± standard error of the mean; n = 6–10; *P < 0.05 versus placebo.

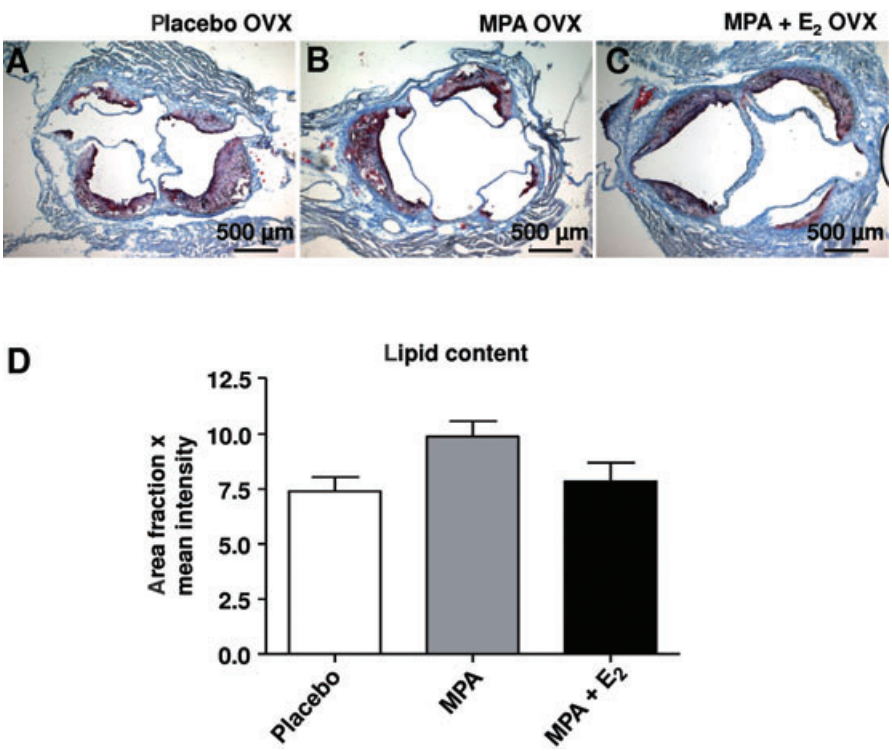

Figure 6 Lipid content of aortic root plaques. (A–C) Aortic root sections stained for lipid deposits from mice treated with placebo, medroxyprogesterone acetate (MPA; 27.7 µg·day^{-1}) and MPA + E_2 (1.1 µg·day^{-1}). (D) Data represent fractions of positively stained areas as determined by digital image analysis; means ± standard error of the mean; n = 5–9.

addition, the frequency of flow-recovery was reduced by MPA and MPA + E_2, which suggests that either the platelet aggregate was more stable or that the adhesion of the platelet thrombus to the carotid artery wall was stronger in MPA-treated OVX mice. A potential explanation of how MPA treatment might cause the increased response to photochemical induced thrombosis would be increased activation of platelets. However, mechanistic experiments revealed that basal platelet activation was not affected by any treatment and that the convulxin-induced P-selectin expression (a marker of platelet activation) was not increased by MPA or MPA + E_2. Thus, platelet activation can be excluded as the underlying cause of the pro-thrombotic state. This is in line with the finding that platelet function in postmenopausal women with CHD is not affected and that *ex vivo* platelet aggregation in response to ADP is even inhibited by both oestrogens and by MPA (Bar *et al.*, 2000). Interestingly, the ETP as a readout for the extent of thrombin generation after addition of exogenous tissue factor was increased in response to MPA, whereas no effect on the ETP was observed after MPA + E_2. Increased thrombin actions on both platelets and fibrinogen in response to MPA might therefore contribute to both increased thrombus formation and thrombus stability. This is in line with clinical results showing that oestrogens + progestins decrease anticoagulant factors such as antithrombin III and protein C (Scarabin *et al.*, 1997; Hoibraaten *et al.*, 2000; Sumino *et al.*, 2005), and increase haemostatic factors in some studies (Scarabin *et al.*, 1997). The result that MPA + E_2 treatment did not affect ETP suggests that E_2 reversed the MPA effect on ETP. Furthermore, it suggests that additional mechanisms are responsible for the observed pro-thrombotic effect of MPA + E_2 treatment.

In search of these additional mechanisms underlying the anti-atherogenic effects of MPA and the prothrombotic effect of MPA and MPA + E_2, plaque composition was determined. MPA + E_2 decreased α-SM-actin accumulation in plaques of the aortic root, which reflects either reduced SMC content and/or reduced differentiation of SMC (Yoshida *et al.*, 2007). These effects on SMC could be of significance, because less SMC accumulation is considered to weaken fibrous caps and de-differentiated SMC are synthetically more active and might therefore support neointimal expansion and luminal narrowing. In addition, HA content was increased in response to MPA + E_2 whereas collagen remained unaffected. HA is a carbohydrate component of the ECM that is thought to support SMC proliferation and migration (Evanko *et al.*, 1999) as well as intimal ECM expansion and is thus likely to be a promoter of luminal narrowing during the course of atherosclerosis (Toole *et al.*, 2002). In addition, HA has been proposed as a pro-thrombogenic matrix component, because dramatic accumulation of HA occurs at the luminal surface of human eroded plaques that caused fatal coronary thrombosis (Kolodgie *et al.*, 2002). Therefore, increased HA content of atherosclerotic plaques might be involved in the increased thrombotic response in the present model especially in response to MPA + E_2. The increase of HA and the decrease of SMC became significant only in the combination group suggesting additive effects of MPA and E_2 on these parameters and possibly providing a mechanism for the increased thrombotic response in the combination group.

In addition to the increased ETP and increased HA accumulation other mechanisms may contribute to the pro-thrombogenic effect of MPA and MPA + E_2 as well. For instance, changes in gene expression within the vascular wall such as increased expression of thrombin receptors in vascular SMC have been described before (Herkert *et al.*, 2001). In addition, down-regulation of endothelial NO synthase in endothelial cells (Zerr-Fouineau *et al.*, 2007) or up-regulation of NADPH oxidase activity (Wassmann *et al.*, 2005) have been reported in response to MPA. Interestingly, MPA has been shown to favour coronary vasospasm in monkeys, which might also confer increased cardiovascular risk (Miyagawa *et al.*, 1997). Taken together, the prothrombotic effect of MPA might be related to increased thrombin formation and/or in the case of the combination treatment due to the remodelling of the arterial wall including increased levels of HA. Future studies are needed to address the role of HA-rich ECM in the arterial responses to MPA and MPA + E_2.

Despite the pro-thrombotic effect of MPA discussed earlier, the present results demonstrated that MPA reduced the extent of atherosclerosis in the present $ApoE^{-/-}$-model by about 20%. It is known that ApoE deficient mice can develop atherosclerosis prior to the development of endothelial dysfunction (Fransen *et al.*, 2008) and that endothelial dysfunction is associated with atherosclerotic plaques (Crauwels *et al.*, 2003). Here we report that endothelium-dependent relaxation in response to ACh was not affected in either treatment group, thereby excluding improvement of endothelial function as a mechanism underlying the anti-atherosclerotic MPA effect. In contrast, vasorelaxation in response to SNAP was significantly impaired after MPA treatment suggesting increased degradation of exogenously formed NO, e.g. through oxidation (d'Uscio *et al.*, 2001). A potential mechanism of the anti-atherosclerotic action of MPA may be that MPA does not only activate progesterone receptors, but also has partial agonistic effects on androgen receptors and glucocorticoid receptors (Poulin *et al.*, 1989; Poulin *et al.*, 1991). The androgenic effect of MPA has been suggested to mediate anti-inflammatory actions during atherosclerosis in postmenopausal women (Wakatsuki *et al.*, 2002). Koubovec *et al.* (2004) showed that MPA reduces cytokine expression in mouse fibroblasts and mediates anti-inflammatory effects through the glucocorticoid receptor. Thus the anti-atherosclerotic effect of MPA detected in the present study might be attributable to both its androgenic and/or glucocorticosteroid properties.

Taken together, the present results on arterial thrombosis in response to MPA in mice are compatible with the clinical findings that HRT with oestrogens and MPA is associated with increased risk of MI and stroke (Hulley *et al.*, 1998; Rossouw *et al.*, 2002; Anderson *et al.*, 2004; Rossouw *et al.*, 2007; Vickers *et al.*, 2007). The present study demonstrated that MPA was strongly pro-thrombotic either alone and in combination with E_2, although atherosclerosis was inhibited. Possible mechanisms include increased thrombin formation and changes in vascular gene expression resulting in altered plaque matrix and SMC phenotype. It might be of clinical interest to evaluate in future studies whether the pro-thrombotic effect can be differentiated from the anti-atherosclerotic effect by use of alternative progestins that

differ from MPA with respect to agonism and antagonism on other steroid receptors.

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Conflicts of Interest

None.

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