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Effects of Oestrogen on Haemodynamic and Vascular Reactivity

A study in animal models and humans

Lisa Brandin

Göteborg 2007

Effects of Oestrogen on Haemodynamic and Vascular Reactivity
– A study in animal models and humans
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To Mum and Dad

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A study in animal models and humans

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Sahlgrenska University Hospital/Östra, Sahlgrenska Academy,
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ABSTRACT

Previous studies have shown that oestrogen, the female sex hormone, plays a protective role in the cardiovascular system. However the site of action remains incompletely understood. Large clinical interventional trials have not proven that longer treatment with oestrogen plus progesterone yields lower incidence of cardiovascular outcomes, suggesting that hormone replacement therapy (HRT) might protect only a selective group of postmenopausal women.

The present study treated normo- and hypertensive female rats and postmenopausal women with oestrogen for short and longer time periods. We also investigated the acute effect of 17β -estradiol on isolated small resistance arteries and the effects of oestrogen treatment on vascular reactivity and endothelial function in a wire-myograph. Further, we recorded haemodynamic parameters during daily life and stress, and evaluated the effect of HRT on the autonomic nervous systems of hypertensive women by evaluating heart rate variability (HRV) with 24 h analysis.

Blood pressure (BP) was attenuated after 24 hour treatment with 17β -estradiol in normotensive postmenopausal women and normo- and hypertensive rats. In hypertensive rats a lowered BP sustained after 10 days of treatment. Although we observed an attenuated heart rate (HR), haemodynamic responses to stress remained largely unaffected. Six months of HRT did not affect BP, HR, HRV, or haemodynamic responses to stress in hypertensive postmenopausal women but did result in reduced sensitivity to noradrenaline, a stress hormone, in subcutaneous arteries. Lower adrenergic response occurred in the resistance arteries of hypertensive rats but not in normotensive rats or women. 17β -estradiol relaxed precontracted mesenteric arteries, due mainly to endothelial release of nitric oxide. We also observed a modulated endothelial response to acetylcholine following 17β -estradiol treatment in normotensive women and hypertensive rats and HRT in hypertensive women.

In conclusion, the effects of oestrogen on vascular reactivity and haemodynamics differed between hypertensive and nonhypertensive subjects and also according to the type of oestrogen used. Decreased BP and HR with 17β -estradiol treatment but not with HRT suggests that 17β -estradiol participates selectively in the haemodynamic system. However, the attenuated adrenergic vascular response observed in hypertensive subjects independent of oestrogen type may contribute to improved blood flow to peripheral tissue even though BP remains unchanged. The clinical importance of the reinforced acetylcholine induced response in normotensive and hypertensive women and rats after oestrogen treatment requires further evaluation.

Key words: adrenergic reactivity, endothelium, haemodynamic, hypertension, oestrogen, postmenopausal women, resistance arteries, spontaneously hypertensive rats, stress

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, identified in the text by their Roman numerals:

- I Brandin L, Gustafsson H. 17beta-estradiol relaxes precontracted mesenteric arteries from male and female rats; a transient effect which is lost after a short incubation period.
In manuscript
- II Brandin L, Bergström G, Manhem K, Gustafsson H. Oestrogen modulates vascular adrenergic reactivity of the spontaneously hypertensive rat.
J Hypertension 2003;21:1695-1702
- III Brandin L, Bergström G, Manhem K, Gustafsson H. Estrogen attenuates ambulatory pressure and heart rate in hypertensive rats with small effects on hemodynamic responses to stress.
Submitted
- IV Manhem K, Brandin L, Ghanoum B, Rosengren A, Gustafsson H. Acute effects of transdermal estrogen on hemodynamic and vascular reactivity in elderly postmenopausal healthy women.
J Hypertension 2003;21:387-394
- V Brandin L, Gustafsson H, Ghanoum B, Milsom I, Manhem K. Chronic effects of conjugated equine estrogen on hemodynamic and vascular reactivity in hypertensive postmenopausal women
Submitted

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ABBREVIATIONS

ACh	acetylcholine
ANOVA	analysis of variance
ANS	autonomic nervous system
AUC	area under curve
BMI	body mass index
BP	blood pressure
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
EDHF	endothelial derived relaxing factor
HF	high frequency power
HR	heart rate
HRT	hormone replacement therapy
HRV	heart rate variability
5-HT	serotonin
KCl	potassium chloride
LF	low frequency power
L-NNA	N(ω)-nitro-L-arginine
MAP	mean arterial pressure
NA	noradrenaline
NO	nitric oxide
OVX	ovariectomy
SBP	systolic blood pressure
SHR	spontaneously hypertensive rat
SP	substance P
TNS	transmural nerve stimulation
TotP	total oscillatory power
VLF	very low frequency power
VSMC	vascular smooth muscle cell
WKY	Wistar Kyoto

INTRODUCTION

Oestrogen, the female sex hormone produced mainly in the ovaries and testes, influences primarily the growth and function of the male and female reproductive systems and also participates in bone maintenance and modifies cardiovascular functions. Observational studies have shown lower incidence of cardiovascular disease (CVD) in premenopausal women compared with age-matched men [Isles 1992] and postmenopausal women [Eaker 1993]. Moreover, hormone replacement therapy (HRT) prevents several CVD risk factors [Nabulsi 1993]. We believed previously that oestrogen protected against cardiovascular events. In early 2000, however, two large prospective interventional trials determined that treatment with conjugated equine oestrogen plus medroxyprogesterone acetate provided no benefit to cardiovascular outcomes in healthy postmenopausal women [Rossouw 2002] or women with established coronary heart disease [Hulley 1998]. Importantly, both trials involved relatively older subjects with a lengthy period of oestrogen deficiency, a limiting factor in their investigations. Data published more recently suggests that HRT may confer cardiovascular benefits only in subjects less than five years from menopause [Brownley 2004].

Women and hypertension

Elevated blood pressure (BP) is a strong and well-recognized risk factor for CVD, and several studies have indicated that female sex hormones protect against hypertension [von Eiff 1986, Iams and Wexler 1979]. However, current debate continues to argue whether increased BP after menopause occurs independently of age [Mueck and Seeger 2004]. Adjusted for other known risk factors that increase with age (body mass index (BMI), reduced physical activity, hypertriglyceridemia, and age itself), postmenopausal women's risk of developing hypertension only moderately exceeds that of premenopausal women [Amigoni 2000]. Although epidemiological studies indicate that HRT slightly reduces elevated BP in postmenopausal women [Amigoni 2000], this finding remains unconfirmed in healthy normotensive postmenopausal women treated with unopposed oestrogen or HRT for three years [The PEP1-trial 1995]. Because oral contraceptives elevate BP in premenopausal women [Woods 1988], hypertensive postmenopausal women did not receive HRT [Mueck and Seeger 2004]. Although studies on the effects of oestrogen in prior hypertensive subjects are sparse, reports that 17β -estradiol prevents the development of high BP in ovariectomized spontaneously-hypertensive rats (SHR) [Gimenez 2006], and that oestrogen even lowers systolic BP, subsequently eased earlier restrictions [Jespersen 1983, Felmeden and Lip 2000]. However, the effect of oestrogen on BP in hypertensive but otherwise healthy subjects needs further investigation.

The effects of oestrogen on vascular reactivity

Several factors likely precede increased peripheral resistance, a consistent feature of experimental [Ferrario and Page 1978] and essential hypertension [Lund-Johansen 1980]. Endothelial dysfunction, characterized by attenuated

relaxation to acetylcholine (ACh), occurs in the conductance and resistance arteries of hypertensive subjects [Watt and Thurston 1989, Konishi and Su 1983, Winquist 1984]. Excessive sympathetic outflow in response to stress may lead to repeated periods of rising BP and trigger hypertrophy in small arteries [Bedi 2000]. Structural changes in the arterial wall likely result in increased contraction in response to vasoconstrictors and impaired relaxation in response to vasodilators [Folkow 1979].

Oestrogen receptors localized in the vascular wall may suggest that this hormone directly affects peripheral arteries and vascular reactivity. Steroid hormone receptors, known as nuclear receptors, participate in transcription or repression of messenger RNA and subsequent protein translation. However, because α and β oestrogen receptors also reside on the cell surface, and thus couple to signalling pathways other than traditional nuclear receptors, rapid and non-genomic effects of oestrogen likely occur [Collins 2001]. Therefore, it is interesting to study both short- and long-term effects of oestrogen on vascular reactivity. Oestrogen increases coronary [Reis 1994] and forearm blood flow in response to reactive hyperemia [Higashi 2001] *in vivo*. Additionally, lowered peripheral resistance observed in hypertensive pre-menopausal women compared to men and postmenopausal women [Messerli 1987] suggests that oestrogen preserves the haemodynamic profile. Although the precise mechanism remains unknown, attenuated contractile response to different agonists [Paredes-Carbajal 1995, Jiang 1992], improved endothelial function [Abou-Mohamed 2003, Collins 1994] and direct effects on vascular smooth muscle cells (VSMC) [Abou-Mohamed 2003, Salom 2002] occurs in larger conductance arteries from animals exposed to oestrogen. In tissue cultures, oestrogen inhibits expression of adhesion molecules on endothelial cells [Caulin-Glaser 1996] and inhibits platelet adhesion [Miller 1994], an action that may prevent atherosclerotic plaque formation and vascular thrombosis. Furthermore, 17β -estradiol limits oxidation of LDL [Keaney 1994], thus preventing atherosclerotic plaque formation and improving endothelial-dependent relaxation in atherosclerotic coronary arteries

In the context of hypertension, it is of great interest to study small resistance arteries where the major pre-capillary drop in pressure occurs and where structural changes in form of hypertrophy and endothelial damage appear [Folkow 1979]. Resistance arteries have a diameter of 100-500 μm and a muscular wall richly innervated by sympathetic constrictor fibres. Because such arteries serve as a tap to the local tissue, structural changes strongly impact flow regulation. Studies of oestrogen's effect on small resistance arteries are scant, but an acute relaxing effect has been reported [Shaw 2000].

Oestrogen and the autonomic nervous system

Hypertension is characterized by increased cardiovascular reaction to stress [Bedi 2000], an augmented haemodynamic response observed also in prior hypertensives [Jern 1983], in men compared to age-matched women [Matthews 1990], and in postmenopausal compared to pre-menopausal women

[Bairey Merz 1998]. Because animal studies show a clear correlation between atherosclerosis development and emotional stress [Manuck 1997], the haemodynamic response to stress *per se* is interesting because BP at rest might be normal. Some studies have postulated that oestrogen protects against cardiovascular hyper-reactivity in normotensive postmenopausal women [Cerinini 2000, Lindheim 1992], spurring interest in studying the effects of oestrogen on hypertensive subjects where a high sympathetic activity is expected.

Since parasympathetic outflow dominates at rest, it may be more equitable to study the effects of oestrogen on BP and HR using ambulatory recordings, as such registration tracks twenty-four hours variations. Further, heart rate variability (HRV) makes possible the study of balance in the autonomic nervous system (ANS) upon heart rate (HR) [Task Force of the European Society of Cardiology 1996]. A 9-year follow-up study on postmenopausal women treated for an acute coronary syndrome showed that some HRV parameters, including total power, very low frequency (VLF) power, low frequency (LF) power, and high frequency (HF) power, independently and significantly predict all-cause mortality [Janszky 2004]. Oestrogen treatment likely results in higher HF power, lower LF%, and lower LF/HF, indicating increased vagal and attenuated sympathetic tone by the female sex hormone [Liu 2003].

No single mechanism likely accounts for oestrogen's effect on the vascular tree, but rather an interaction on different components involved in atherosclerotic formation, the haemodynamic system, and vascular tone. Further, acute and chronic exposure to oestrogen as well as the type of oestrogen used most likely interacts with these factors in different ways. The effects of oestrogen in experimental studies *in vitro* are not necessarily reproduced in the presence of a whole system's confounding factors. Most importantly, the differences between species argue that the site of action might be unequal. After all, animals are animals and women are human beings.

AIMS

Against this background, the aims of this study included:

- Investigating the acute effects of oestrogen on isolated small resistance arteries from rats, taking into account gender, dose, and type of exposure.
- Studying the effects of oestrogen treatment on vascular reactivity and endothelial function in normo- and hypertensive female rats and postmenopausal women.
- Investigating the effects of oestrogen on haemodynamic parameters during daily life and stress in normo- and hypertensive female rats and postmenopausal women.
- Testing the hypothesis that long-term oestrogen treatment alters the autonomic cardiovascular function towards increased vagal activity in hypertensive postmenopausal women.

MATERIALS

Subjects and study design

Paper I

We investigated the acute effects of increasing concentrations of 17 β -estradiol on noradrenaline (NA: 5 μ mol/l) precontracted mesenteric arteries in male and reproductive female Wistar rats, and further evaluated the mechanisms behind a plausible effect by blocking the competitive inhibitor of nitric oxide synthase with N(ω)-nitro-L-arginine (L-NNA: 100 μ mol/l), depolarizing the smooth muscle cell membrane with potassium chloride (KCl: 25 mmol/l) and blocking the cyclo-oxygenase pathway with indomethacin (10 μ mol/l) in stated order. In addition, we studied the subacute effects of low and high doses of 17 β -estradiol in mesenteric arteries from male rats. Before performing dose-response curves of three different agonists, arteries were incubated (30 min) with placebo or 17 β -estradiol (10⁻⁹ and 10⁻⁷ mol/l). Two agonists, NA (0.08 – 10 μ mol/l) and serotonin (5-HT: 0.02 – 1.25 μ mol/l), act through receptors on VSMC, and KCl (4 – 125 mmol/l) depolarizes the VSMC membrane and causes a direct vascular contraction.

Paper II

Reproductive female SHR and normotensive Wistar Kyoto (WKY) rats were allocated randomly to either a control group (group C) or groups that underwent ovariectomy (group OVX) or ovariectomy combined with oestrogen supplementation (17 β -estradiol, 150 μ g/kg per day) for either 1 day (group acute E2) or 10 days (group 10E2). Ovariectomy was performed during anaesthesia and 17 β -estradiol (dissolved in sesame oil to a final concentration of 0.15 mg/ml) was administered daily by subcutaneous injection during the morning hours (8 a.m. to 11 a.m.). Contractile properties to transmural nerve stimulation (TNS: 0.12 – 32 Hz) and exogenous NA (0.08 – 10 μ mol/l) were performed on mesenteric arteries *in vitro*. Endothelial function was analysed by applying ACh (10⁻⁹ – 10⁻⁶ mol/l), which releases nitric oxide (NO) in this type of vessel, in increasing concentration to NA (5 μ mol/l) precontracted arteries. In a complementary series, cumulative frequency response curves of TNS were constructed on mesenteric arteries from sham operated (Csham-SHR and Csham-WKY), ovx-SHR and ovx-WKY in the absence and presence of the α_1 -adrenergic antagonist prazosin (1 μ mol/l).

Paper III

Ambulatory mean arterial pressure (MAP), HR, and general activity were measured for 24 hours in six female SHR and WKY rats. Recordings were performed in intact rats (intact), following ovariectomy (10 days) (ovx), placebo-injection (2 days), and 17 β -estradiol injection (150 μ g/kg per day) for 1 day (acute E2) and after 10 days (10E2). Each rat acted as its own control for paired observations. Ovariectomy and injection of placebo (sesame oil, 0.1ml/100g) and 17 β -estradiol were performed as described in *Paper II*. At

the conclusion of each treatment period, we exposed the rats to a short period of stress. We monitored MAP and HR continuously before, during, and after stress provocation.

Paper IV

A randomized double-blind, cross-over, placebo-controlled study on eleven healthy postmenopausal women recruited from the population-based study BEDA. All subjects were non-smokers and had low cardiovascular risk with no signs of hypertension, diabetes (fasting blood glucose < 5.0 mmol/l), or hyperlipidemia (fasting total cholesterol < 6 mmol/l). The short term effects of 17 β -estradiol on resting and mental stress evoked HR, office systolic (SBP), and diastolic (DBP) blood pressure. In addition, we evaluated 24 h ambulatory BP and HR. Subjects (mean age 67 years; range 66-72 years) were postmenopausal for at least 10 years and none had received any kind of hormonal substitution for the prior six months. Transdermal 17 β -estradiol (100 μ g/24 h) and placebo were administered for 24 hours before recordings. Blood samples were collected for analysis of P-adrenaline, P-noradrenaline, and P-estradiol. Office BP and HR were measured, and subjects participated in a stress test. Gluteal fat biopsies were taken from seven subjects, small subcutaneous arteries (diameter 383 ± 27 μ m) were mounted in a Multi Myograph, and contractile properties to NA (0,08 - 10 μ mol/l) and KCl (4 - 125 mmol/l) were analysed. The endothelium was further analysed by applying ACh (10^{-9} - 5×10^{-6} mol/l) and substance P (SP: 10^{-12} - 5×10^{-9} mol/l) to NA (10 μ mol/l) precontracted arteries before and after incubation with L-NNA (300 μ mol/l). We also tested ACh-induced relaxation during a slight potassium-induced depolarisation (KCl: 15 mmol/l) and after incubation with indomethacin (1 μ mol/l).

Paper V

A randomized, double-blind, cross-over, placebo-controlled study on 20 hypertensive but otherwise healthy postmenopausal women recruited from an advertisement in the local newspaper in Göteborg/Sweden. Subjects were non-smokers and had no history of thromboembolic disease, diabetes, hyperlipidemia, or alcohol abuse; subjects were included if they performed normal ECG; two BP-measurements $\leq 170/100$; normal measurements of p-APC-resistance, p-protein C and S; and normal physical, mammography, and gynaecological examinations. We evaluated the long-term effects of HRT on HR, SBP and DBP at rest and during mental stress. We performed ambulatory 24-hour BP and HR recordings, and further analysed the effect of oestrogen on autonomic control with spectral and time domain analysis of HRV. Subjects (mean age 56 years; range 53-61 years) were postmenopausal for at least 2 years and none had received any kind of hormonal substitution for the prior three months. The women included were treated with 1-4 antihypertensive drugs (beta-blockers, calcium channel blockers, ACE-inhibitors, angiotensin receptor blockers, and diuretics), and we aimed for a normalized office BP. Conjugated oestrogen, Premelle® (conjugated oestrogen USP 0.625 mg and medroxyprogesteronacetat 5 mg), or placebo were administered daily for six

months before beginning the experiment. We collected blood samples for s-estradiol, s-testosterone, and s-SHBG analysis and measured office BP at baseline and after six and twelve months of therapy. Subjects performed a mental stress test in the laboratory. Ambulatory BP, HR and HRV recordings were registered outside the hospital. We analysed subcutaneous arteries (diameter $464 \pm 33 \mu\text{m}$) from 17 subjects using the wire-myograph technique. The contractile properties to NA ($0.02 - 10 \mu\text{mol/l}$), KCl ($4 - 125 \text{ mmol/l}$), and TNS ($0.02 - 16 \text{ Hz}$) in the presence of cocaine ($1 \mu\text{mol/l}$) and propranolol ($1 \mu\text{mol/l}$) were analysed. Further, the endothelium was analysed by applying ACh ($10^{-9} - 5 \times 10^{-6} \text{ mol/l}$) and SP ($10^{-12} - 5 \times 10^{-9}$) to NA ($10 \mu\text{mol/l}$) precontracted arteries before and after incubation with L-NNA ($300 \mu\text{mol/l}$) and two Ca^{2+} -sensitive K^{+} -channel-blockers (charybdotoxin $0,1 \mu\text{mol/l}$ and apamin $0,7 \mu\text{mol/l}$).

METHODS

Myograph technique

In animal studies, the rats were anaesthetised and subsequently sacrificed by excision of the heart (*Papers I and II*). The mesentery was removed and small arteries from the second or third branch of the arcades feeding the jejunum (internal diameter approx. 200 μm) were used for *in vitro* studies of vascular reactivity.

In the human studies, (*Papers IV and V*), gluteal fat biopsies (covering 1 x 0.5 x 1 cm of subcutaneous tissue) were taken under local anaesthesia and small subcutaneous arteries were dissected free from underlying tissue.

We investigated contractile properties and endothelial function of resistance arteries using the wire-myograph technique (Multi-myograph 610 M, Danish Myo Technology Aarhus Denmark) [Mulvany and Halpern 1977]. Small arteries were dissected free from connective tissue and mounted on two parallel stainless steel-wires in a small organ bath (volume 8 ml) (Figure 1). The wires were clamped onto two supports, i.e., one support was attached to a force transducer and the other to a micrometer that adjusts vessel distension. Great care was taken during the mounting procedure in order not to harm the thin endothelial layer lining the lumen or the nerve-endings in the adventitia, close to VSMC. The contractile responses of the small arteries were measured as tension (i.e., developed force/mm vessel wall length) under isometric conditions. To minimize differences in intrinsic force production and sensitivity to different agonists dependent on the degree of stretch, we normalized the arteries' lumen diameters before beginning the experiment [Mulvany 1982]. We extended each vessel stepwise and measured the wall tension. The data was fitted to an exponential curve, and the effective pressure (P_i) needed to stretch the vessel to a normalized internal circumference (IC), i.e., expected diameter of relaxed vessel exposed to 100 mmHg transmural pressure (IC_{100}), was calculated using Laplace equation.

$$P_i = \text{Wall tension} / (IC/2\pi)$$

The vessels were set to 0.9 IC_{100} , the point of maximal force production in mesenteric small arteries [Mulvany and Halpern 1977]. Prior to the experimental protocol, vessels were equilibrated (30 min) and thereafter contracted maximally with NA (10 $\mu\text{mol/l}$) and KCl (125 mmol/l). We considered relaxation by ACh (10 $\mu\text{mol/l}$) on a precontracted artery as proof of an intact endothelium.

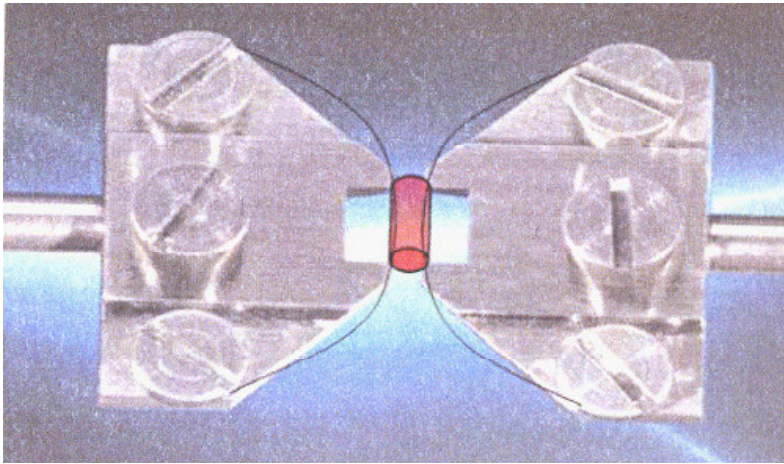


Figure 1. Illustration of a vessel mounted in a wire-myograph

We performed TNS by electrical field stimulation (*Papers II and V*). Two platinum electrodes were placed on either side of the vessel and constant current pulses of 85mA with altering polarity were passed between (duration 2 ms, in human arteries and a complementary series 0.1 ms). Because tetrodotoxin (0.1 $\mu\text{mol/l}$) blocked the ensuing contraction completely, we considered the contractions neurogenic [Nilsson and Folkow 1982]. A cumulative frequency-response relationship to TNS was used as the purinergic components diminish progressively with stimulation and therefore mainly represent the adrenergic component of contraction [Nilsson and Folkow 1982]. To avoid neuronal re-uptake and β -adrenergic effects of NA, we added cocaine (1 $\mu\text{mol/l}$) and propranolol (1 $\mu\text{mol/l}$) to the bath. We maintained each frequency (0.12 - 32 Hz) until a stable tension was reached (duration about 30 - 60 sec). The vessels exposed to TNS were not used again in the experiment.

Haemodynamic recordings

We implanted a radiotelemetry transmitter containing a fluid-filled intra-arterial catheter inserted into the aorta, and a sensor secured to the abdominal muscle (Data Sciences International, Inc., St Paul, Minnesota, USA) of anaesthetised female rats (*Paper III*) and secured it to the abdominal muscle. The rats were placed in individual cages containing a receiver plate, and the signal was collected using the Dataquest LabPRO Acquisition System (version 3.0, Data Sciences International, Inc.). Rats were allowed to recover for five days before experimental set-up. We measured MAP, HR, and general

activity continuously for 24-hour periods, with a 12-second sampling period (500 Hz) at the end of each treatment period. Based on pooled activity data, we divided the 24-hour period into passive (12 a.m. to 5 p.m.) and active (5 p.m. to 12 a.m.) periods.

In the human studies (*Papers IV and V*), we recorded supine office BP after 30 min rest. Ambulatory BP (Spacelab 90202) and HR (ASPECT Holter System, Danica Biomedical AB, Borlänge, Sweden), was performed three times/h (6 a.m. to 12 p.m.) and twice/h (12 p.m. to 6 a.m.) outside the hospital, and subjects were told not to change their normal daily activities.

Stress-experiments

In the animal study (*Paper III*), we monitored baseline values of MAP and HR and then exposed rats to acute stress, i.e., removing them singly from their cages and blowing a jet of air on the nose (15 sec) while holding their tails. MAP and HR were registered during and after (10 min) the stress procedure.

In the human studies (*Papers IV and V*), we registered baseline values of BP and HR after the subjects rested in a supine position (10 min). Subjects then performed a mental arithmetic test while undergoing registration of BP and HR every minute (Spacelab 90202, Redmond, Washington, USA). During the stress period, subjects were asked to subtract the number 7 sequentially from 100 (e.g., $100 - 7 = 93$; $93 - 7 = 86$; etc.) apace with a metronome and distracted by a bell. Finally, BP and HR measurements continued every minute while subjects rested alone in a quiet room.

Spectral analysis

To further analyse the effect of HRT on autonomic control, we performed HRV time- and frequency-domain analysis (*Paper V*). Subjects underwent Holter monitoring (24 h) using a 3-channel amplitude modulated tape recorder (ASPECT Holter System, Danica Biomedical AB, Borlänge, Sweden) with three leads (modified inferior, CM1, and CM5); we used the ASPECT Holter System (Barlett) analysis programme for tape analysis. The frequency histogram of normal R-R intervals was displayed, and electrocardiogram strips of the interval of R-R distribution were visually checked. Additionally, we analysed a histogram of the consecutive R-R ratio, excluding from further analysis cycles 80 - 120 % greater than the preceding R-R intervals. This preliminary analysis allowed exclusion of artefacts, premature beats, or postextrasystolic pauses. All tapes were subsequently analysed to measure HRV using Welsh programme. Time-domain measurements of HRV calculated from 24-hour electrocardiographic recordings included the mean of all normal RR-intervals (mean-NN); standard deviation of all normal R-R intervals (SDNN); standard deviation of averaged RR intervals of all 5-min segments (SDANN); the square root of the mean of squared differences between adjacent successive RR intervals (rMSSD); and the percentage of difference

between adjacent normal RR intervals > 50 ms (pNN50). Spectral power measurements were computed by Welch (FFT) transform analysis. We calculated total oscillatory power (TotP) in the overall signal (0.003 to 0.400 Hz), and used spectral plots to identify three subsets of the frequency domain: low frequency power (LF: 0.040-0.150 Hz); high frequency power (HF: 0.150-0.400 Hz); and very low frequency power (VLF: 0.003-0.040 Hz).

Statistical analysis

Subjects were used as their own controls when appropriate.

In myographic studies, concentration-response relations were analysed by non-linear regression (Graph Pad Systems, San Diego, California, USA). The curves were fitted to the individual concentration-response data based on the relationship $E = E_{\max} \frac{A^P}{(A^P + EC_{50}^P)}$, where E is the response obtained for a given concentration A, E_{\max} is the maximally attainable response, EC_{50} is the concentration required for half-maximal effect and exponent P represents the slope of the relationship (Hill coefficient). We used Graph Pad Systems to calculate the area under the curve (AUC). Statistical analysis was performed with one-way ANOVA for repeated measures and Student's *t* test for paired and unpaired observations. To evaluate treatment effects within the strains, we used two-way ANOVA for repeated measures.

Office BP, average values of the different segments (pre-stress, stress and post-stress) of the mental stress test (*Papers III, IV and V*), and plasma analysis of biochemical data were analysed by ANOVA for repeated measures.

Ambulatory monitoring data (*Papers III, IV and V*), calculated as 1-h means of HR, SBP, DBP, and MAP, were analysed by two-way ANOVA for repeated measurements, taking into account subjects, treatment, and time.

RESULTS

17 β -estradiol relaxes precontracted mesenteric arteries from male and female rats (*Paper I*)

Acute application of 17 β -estradiol (10^{-7} and 10^{-6} mol/l) relaxed NA precontracted isolated mesenteric small arteries from male and female rats (Figure 2) ($p < 0.05$). The maximum response to 17 β -estradiol did not differ between genders. A small relaxation observed at lower concentrations of 17 β -estradiol (10^{-9} and 10^{-8} mol/l) in male arteries did not attain statistical significance (Figure 2a). Relaxation to 17 β -estradiol was immediate and the underlying mechanisms differed depending on gender. In male arteries, L-NNA incubation completely inhibited 17 β -estradiol induced relaxation, suggesting that 17 β -estradiol releases NO from the endothelium. On the other hand, L-NNA alone did not fully inhibit relaxation induced by the highest 17 β -estradiol concentration in female arteries, suggesting an additional mechanism besides the NO-dependent pathway. Adding low dose potassium totally suppressed artery relaxation, indicating that the acute effect of high 17 β -estradiol doses in female arteries resulted from a direct hyperpolarizing effect on VSMC or a concomitant release of an endothelial-derived hyperpolarizing factor (EDHF) together with NO. Indomethacin had no further effect.

Incubation of isolated vessels with 17 β -estradiol before adding a contractile agent to the bath has also been described as acute in literature; therefore, we tested this experimental setup in small arteries. Following incubation (30 min) of mesenteric arteries from male rats with low (10^{-9} mol/l) and high (10^{-7} mol/l) concentrations of 17 β -estradiol, we performed dose-response relationships to NA, 5-HT, and KCl. Incubation with 17 β -estradiol did not affect the sensitivity and maximal contraction of either agonist compared to vehicle.

The effects of oestrogen on endothelial function (*Papers II, IV and V*)

The maximal relaxation to ACh in NA-precontracted small mesenteric arteries from female intact and ovx-SHR showed impairment compared to arteries from corresponding WKY rats (Figure 3) ($p < 0.01$) (*Paper II*). High concentrations of ACh ($> 10^{-7}$ mol/l) caused mesenteric artery contraction in SHR but further relaxation in WKY. 17 β -estradiol treatment (acute E2 and 10 E2) attenuated this paradoxical contraction and substantially improved relaxation compared to intact and ovx-SHR (Figure 3b) ($p < 0.05$).

We analysed endothelial function of subcutaneous arteries from postmenopausal women using two different agonists, ACh and SP (*Papers IV and V*). Both agonists relaxed NA-precontracted arteries substantially. Following 17 β -estradiol treatment or HRT, relaxation capacity did not differ in either normotensive or hypertensive women, respectively, compared to placebo. In-

Fig. 2a Male

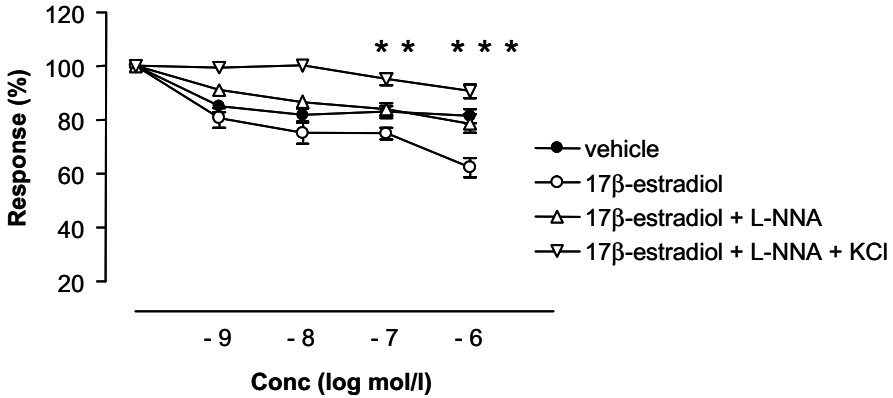


Figure 2. Cumulative dose-response curves for the effects of 17β-estradiol on precontracted small arteries from male (a) and female (b) Wistar rats before and after incubation with either 100 μmol/l N(ω)-nitro-L-arginine (L-NNA) or 100 μmol/l L-NNA and 25 mmol/l potassium chloride (KCl). Vehicle is not containing 17β-estradiol. Responses are expressed as percentage of the submaximal response to noradrenaline. *p<0.05, **p<0.01, ***p<0.001 for 17β-estradiol induced relaxation compared to vehicle. Data are expressed as means ± SEM.

Fig. 2b Female

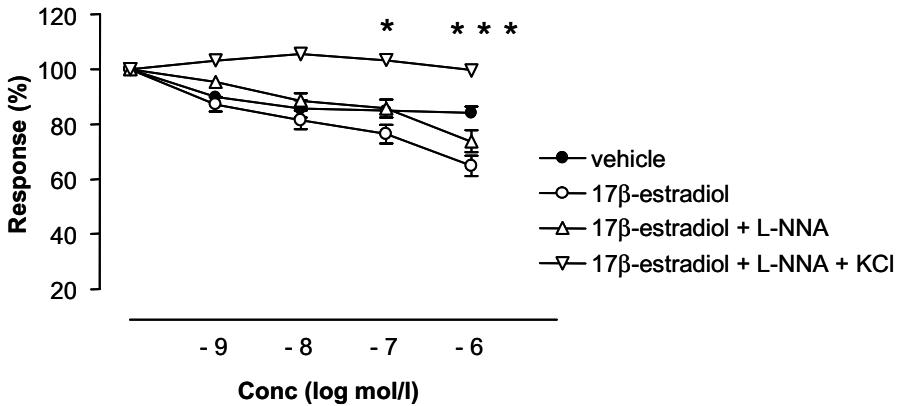


Fig. 3a WKY - Acetylcholine

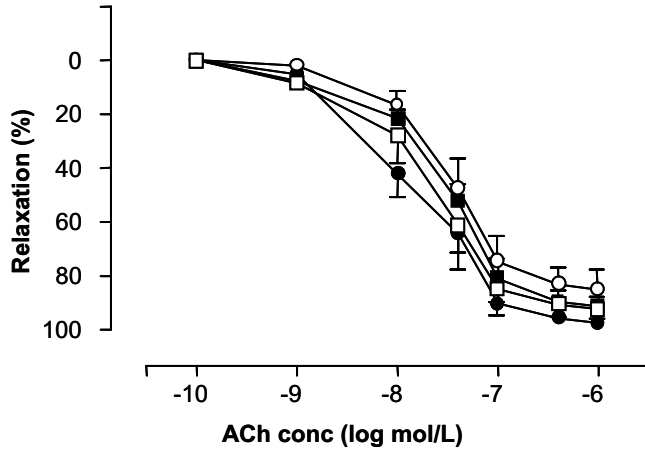
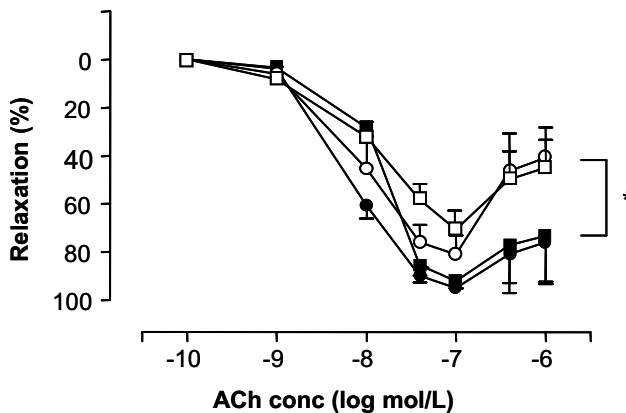


Figure 3. Cumulative dose-response relations to acetylcholine (ACh) for precontracted mesenteric small arteries from female normotensive WKY rats (a) and spontaneously hypertensive rats (SHR) (b) undergoing different treatments: intact (open square), ovariectomy (open circle), ovariectomy and 17 β -estradiol treatment for one day (filled square) and 10 days (filled circle). Relaxation is expressed as percent of maximal response to noradrenaline. AUC * $p < 0.05$. Data are expressed as means \pm SEM.

Fig. 3b SHR - Acetylcholine



dependent of treatment in normotensive ($p < 0.01$) and hypertensive (Figure 4) ($p < 0.001$) women, L-NNA significantly inhibited SP-induced relaxation, suggesting that SP releases NO from the endothelium. On the other hand, L-NNA did not affect ACh-induced relaxation significantly in normotensive (*Paper IV*) or HRT-treated hypertensive women (Figure 5b) (*Paper V*). Thus, ACh likely releases another relaxing compound such as EDHF or prostacyclin from the endothelium in subcutaneous arteries. A small unspecific depolarization with potassium in combination with L-NNA attenuated relaxation to ACh in arteries of normotensive postmenopausal women treated with placebo ($p < 0.001$); relaxation after 17β -estradiol treatment remained unaffected. Prostanoids, inhibited with indomethacin, did not contribute to relaxation (*Paper IV*). Incubation with L-NNA (*Paper V*) resulted in a moderately attenuated response to ACh in placebo-treated hypertensive women (Figure 5a) ($p < 0.01$), indicating a small release of NO in oestrogen-deprived hypertensive women. Thereafter, we incubated the arteries with two potassium channel blockers that more specifically inhibited relaxation due to EDHF. Potassium channel blockers combined with L-NNA reduced sensitivity to ACh in both placebo- ($p < 0.01$) and HRT-treated ($p < 0.05$) arteries of hypertensive postmenopausal women (Figure 5) (*Paper V*). However, NO and EDHF inhibition was more successful after placebo treatment compared to HRT ($p < 0.01$).

Oestrogen modulates vascular adrenergic reactivity (*Papers II, IV and V*)

The maximal adrenergic response to TNS of small mesenteric arteries from SHR and WKY was approximately 60 - 75 % of the maximal NA-induced contraction and did not differ between strain or treatment groups (*Paper II*). We observed no difference in sensitivity to TNS (0.12 - 32 Hz) between intact female WKY and SHR. However, ovariectomy yielded a greater vascular response to TNS in SHR compared to WKY (Figure 6a) ($p < 0.05$). Increased sensitivity to TNS in ovx-SHR attenuated after only one day of 17β -estradiol treatment; consequently, the dose response relationships to TNS did not differ significantly between WKY and SHR (acute E2). Dose response curves to TNS were identical (10 E2) after ten days of 17β -estradiol treatment (Figure 6b). In a complementary study, we divided SHR and WKY female rats randomly into two groups; one group underwent a sham operation and the other ovx. An increased vascular response to TNS in the ovx-SHR was repeated but we observed no difference between Csham-WKY and Csham-SHR. In the presence of prazosin (1 μ mol/l), an α_1 -adrenergic antagonist, contraction to TNS decreased significantly (50 - 75%) and no longer differed between ovx-WKY and ovx-SHR (Figure 7). Sensitivity to exogenously applied NA was similar between strains, but we observed a tendency towards increased maximal contractile response (E_{max}) in mesenteric arteries from SHR compared to WKY, independent of treatment ($p = ns$).

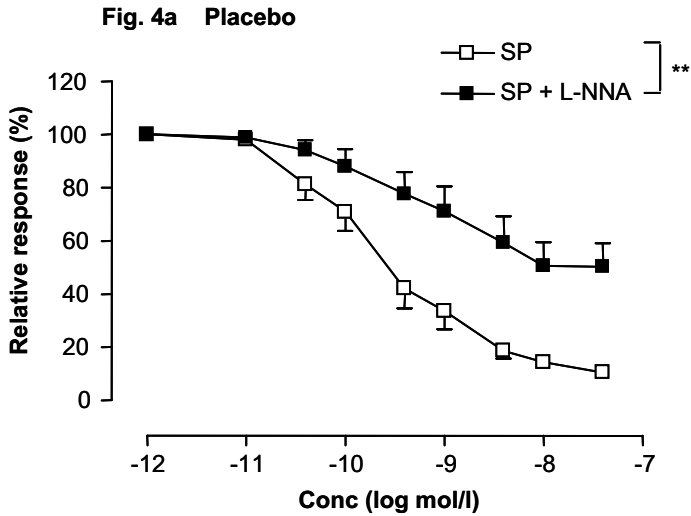
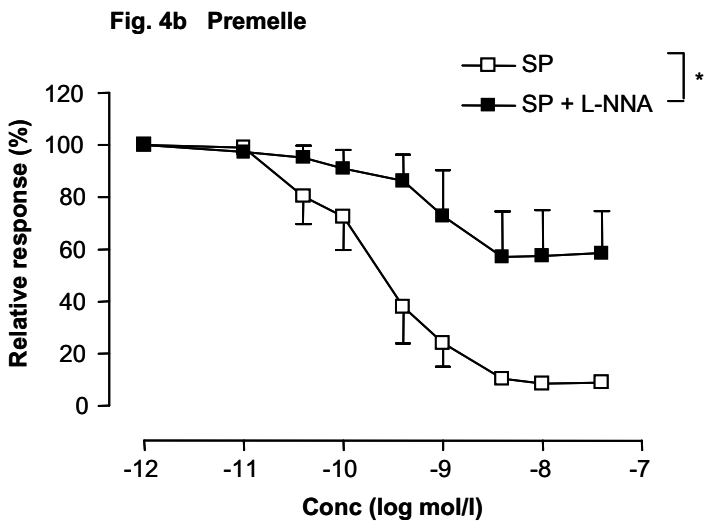


Figure 4. Cumulative dose-response relations to Substance P (SP) for precontracted subcutaneous small arteries from hypertensive postmenopausal women after 6 months of treatment with placebo (a) and Premelle® (b) before and after incubation with 300 μ mol/l N(w)-nitro-L-arginine (L-NNA). Relaxation is expressed as percent of maximal response to noradrenaline. * $p < 0.05$, ** $p < 0.01$. Data are expressed as means \pm SEM.



We also observed an attenuated response to TNS in subcutaneous arteries from hypertensive women following HRT-treatment, compared to baseline ($p < 0.01$) and placebo ($p < 0.05$) (Figure 8a) (*Paper V*). Similar to rat mesenteric arteries, the maximal response to TNS was approximately 60 - 75% of the maximal NA-induced contraction and did not differ with treatment. However, the dose response curve to NA shifted significantly to the right after HRT compared with baseline and placebo (Figure 8b) ($p < 0.05$), suggesting reduced sensitivity to NA at the receptor level in hypertensive postmenopausal women. Maximal response to NA was unaffected.

Although, *Paper IV* did not evaluate response to TNS, 17β -estradiol-treatment (24 h) did not affect sensitivity or maximal contraction to exogenously-applied NA in normotensive postmenopausal women. Further, neither 17β -estradiol treatment nor HRT affected the contractile properties to potassium-induced unspecific depolarization (*Papers I, II, IV* or *V*), suggesting that oestrogen does not influence VSMC contractile properties *per se*.

17β -estradiol attenuates blood pressure (*Papers III, IV* and *V*)

SHR showed higher MAP than WKY, independent of oestrogen status (*Paper III*). Ovariectomy lowered ambulatory MAP in WKY ($p < 0.001$) but did not affect MAP in SHR compared to intact rats (Figure 9) (*Paper III*). In both ovx-WKY ($p < 0.05$) and ovx-SHR ($p < 0.001$), a single injection of 17β -estradiol resulted in a small but significant decrease in MAP within a few hours (Figure 9) (*Paper III*). In addition, normotensive postmenopausal women treated with transdermal 17β -estradiol (24 h) showed reduced ambulatory SBP ($p = 0.05$) and DBP ($p < 0.05$) compared to placebo (*Paper IV*).

Decreased MAP was sustained in ovx-SHR after 10 days of 17β -estradiol treatment (Figure 9b) ($p < 0.001$), and we observed a slight but statistically significant increase in MAP in ovx-WKY during the active period of the day (10 E2) ($p < 0.01$). Six months of HRT did not affect ambulatory BP compared to baseline or placebo in hypertensive postmenopausal women receiving antihypertensive medication (*Paper V*).

BP increased significantly in response to stress in both humans and rats (*Papers III, IV*, and *V*), and 17β -estradiol treatment yielded only small benefit (*Papers III* and *IV*). We observed decreased DBP and unaltered SBP during and after stress in normotensive women after 24 hours of 17β -estradiol treatment compared to placebo ($p < 0.01$) (*Paper IV*). In the animal model, SHR peaked higher than MAP during stress compared to WKY, independent of treatment, but statistically greater MAP reactivity occurred only in intact SHR compared to WKY ($p < 0.001$). In ovx rats, 17β -estradiol did not affect peak MAP, MAP reactivity during stress, or MAP recovery after stress (*Paper III*). Six months of HRT did not influence BP response to stress in hypertensive postmenopausal women (Figure 10) (*Paper V*).

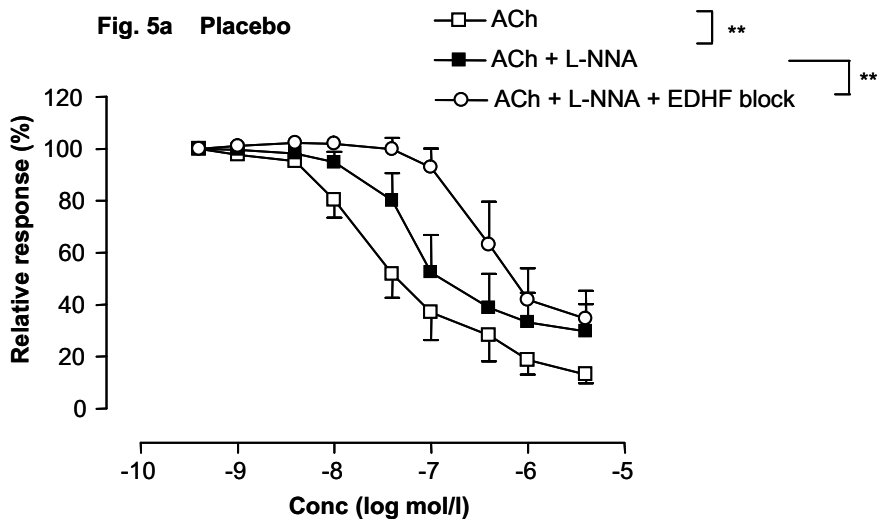


Figure 5. Cumulative dose-response relations to acetylcholine (ACh) for precontracted subcutaneous small arteries from hypertensive postmenopausal women after six months of treatment with placebo (a) and Premelle® (b) before and after incubation with either 300 $\mu\text{mol/l}$ N(ω)-nitro-L-arginine (L-NNA) or 300 $\mu\text{mol/l}$ L-NNA, charybdotoxin 0,1 $\mu\text{mol/l}$ and apamin 0,7 $\mu\text{mol/l}$ (block EDHF). Relaxation is expressed as percent of maximal response to noradrenaline. * $p < 0.05$, ** $p < 0.01$. Data are expressed as means \pm SEM.

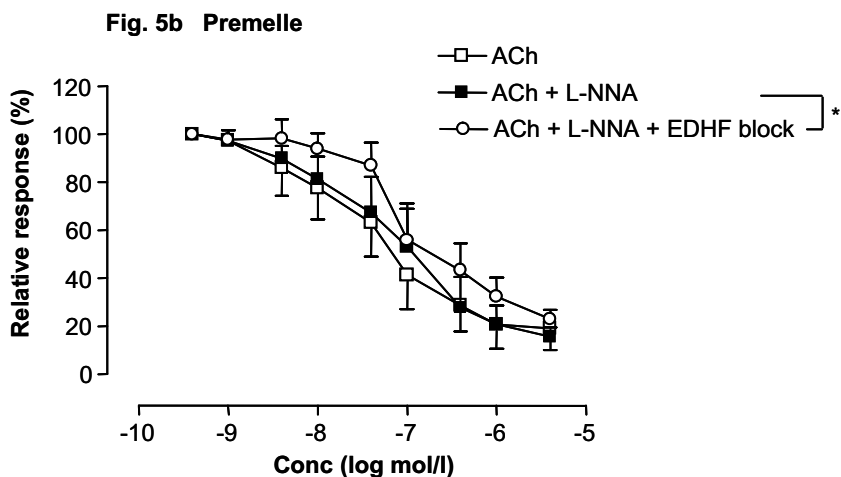


Fig. 6a Nerve stimulation

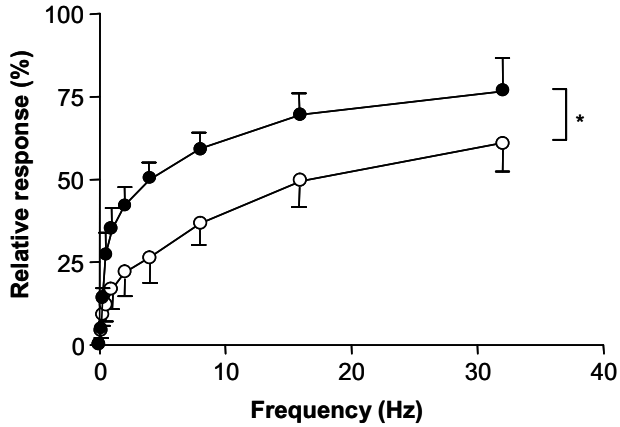


Figure 6. Cumulative frequency-response curves for the effects of transmurial nerve stimulation of mesenteric arteries from female normotensive WKY rats (open circle) and spontaneously hypertensive rats (SHR) (filled circle) after ovariectomy (ovx) (a) and treatment with 17β -estradiol for ten days (10 E2) (b). Responses are expressed as percentage of the maximal response to exogenous noradrenaline. AUC $*p < 0.05$. Data are expressed as means \pm SEM.

Fig. 6b Nerve stimulation

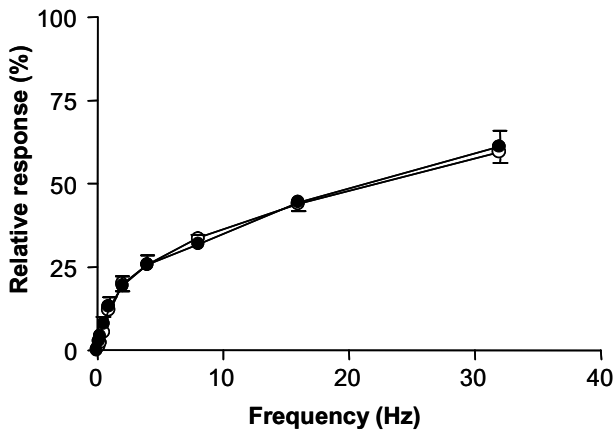


Fig. 7 Nerve stimulation

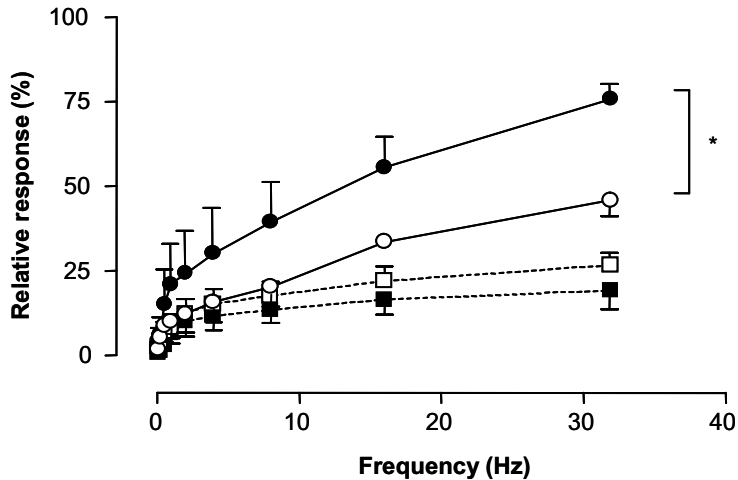


Figure 7. Cumulative frequency-response curves for the effects of transmural nerve stimulation of mesenteric arteries from ovariectomized female normotensive WKY rats (open symbols) and spontaneously hypertensive rats (SHR) (filled symbols), before (circles) and after incubation with prazosin (α_1 -receptor blockade) (square symbols). Responses are expressed as percentage of the maximal response to exogenous noradrenaline. AUC * $p < 0.05$. Data are expressed as means \pm SEM.

17 β -estradiol attenuates heart rate (*Papers III, IV and V*)

Independent of oestrogen status, HR in WKY exceeded that of SHR (*Paper III*). After 10 days of 17 β -estradiol treatment, HR decreased considerably in ovx-WKY (45 beats/min in passive period ($p < 0.001$), 4 beats/min in active period ($p = ns$)) and ovx-SHR (29 beats/min in passive period ($p < 0.001$) and 34 beats/min in active period ($p < 0.001$)) (Figure 11) (*Paper III*). However, ovariectomy *per se* minimally affected HR, suggesting that other ovarian hormones counteract the effect of oestrogen or that HR reduction requires high doses of exogenously-applied 17 β -estradiol. However, we observed a small HR decrease in SHR during the passive period following ovx ($p < 0.001$). 17 β -estradiol-treatment (24 h) reduced HR in normotensive postmenopausal women during the morning hours (6 – 8 beats/min ($p < 0.01$)) (*Paper IV*) but not in ovx female rats. Postmenopausal women showed no effect on HR after six months HRT (*Paper V*).

Fig. 8a Nerve stimulation

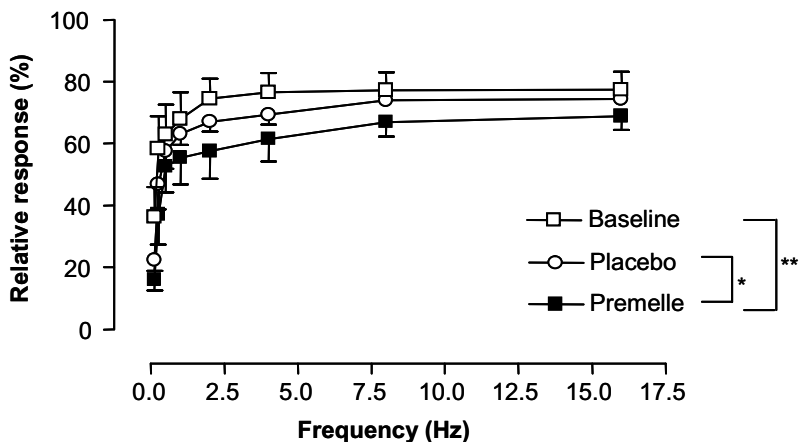
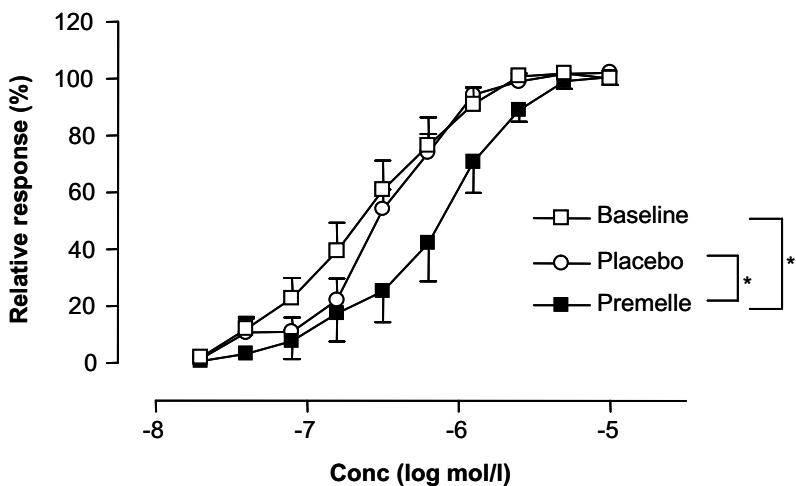


Figure 8. Cumulative frequency-response curve for the effects of transmural nerve stimulation (a) and dose-response relations to exogenous noradrenaline (b) for subcutaneous small arteries from hypertensive postmenopausal women at baseline, after six months of treatment with placebo and Premelle®. Responses are expressed as percentage of the maximal response to exogenous noradrenaline. * $p < 0.05$, ** $p < 0.01$. Data are expressed as means \pm SEM.

Fig. 8b Noradrenaline



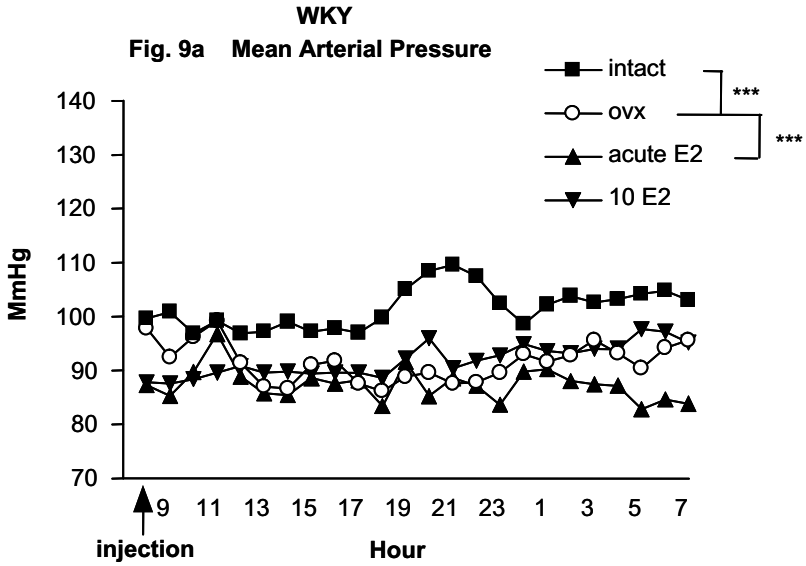
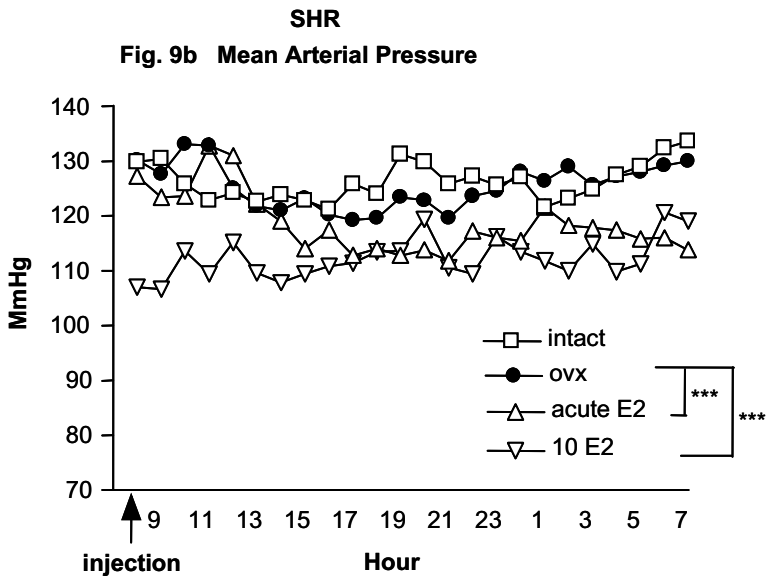


Figure 9. Mean arterial pressure for 24 hours in female normotensive WKY rats (a) and spontaneously hypertensive rats (SHR) (b), each rat acted as its own control. Intact, ovariectomy (ovx), 17 β -estradiol treatment for one day (acute E2) and ten days (10 E2). Passive period (ranging from 12 - 17) and active period (the remaining of the day) based on pooled activity data. Arrow shows time for injection. *** $p < 0.001$. Data are expressed as means.



Stress increased HR significantly in both humans and rats (*Papers III, IV and V*). Oestrogen treatment affected neither stress-induced HR reactivity nor HR recovery in postmenopausal women (*Papers IV and V*) or normotensive female rats (*Papers III*). However, while 17 β -estradiol treatment (24 h) generated slightly lower HR reactivity in ovx-SHR ($p < 0.01$), treatment extended over 10 days yielded increased reactivity compared with untreated ovx-SHR ($p < 0.05$). The highest HR during stress occurred in intact SHR ($p < 0.001$) and 17 β -estradiol treatment did not affect peak HR. HR reactivity was greater in intact and ovx-SHR compared to corresponding WKYs ($p < 0.05$), but we observed no difference between strains after treatment with 17 β -estradiol.

Heart rate variability (*Paper V*)

Six months of HRT in 18 postmenopausal women receiving antihypertensives did not significantly affect time domain or spectral analysis of HRV compared to baseline or placebo (*Paper V*).

Fig. 10a Systolic Blood Pressure

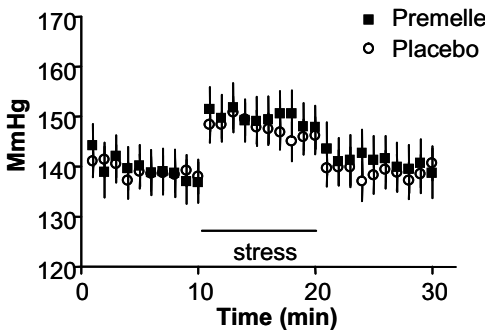


Figure 10. Systolic blood pressure (a) and diastolic blood pressure (b) in hypertensive women in response to mental stress after six months of treatment with placebo and Premelle®. Data are expressed as means \pm SEM.

Fig. 10b Diastolic Blood Pressure

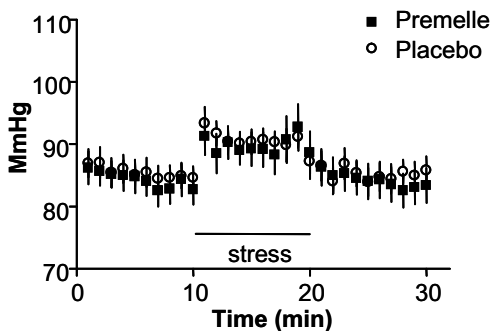


Fig. 11a WKY - Heart Rate

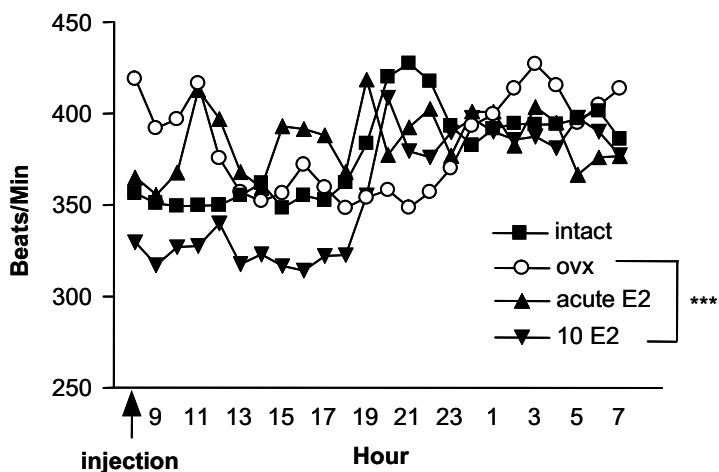
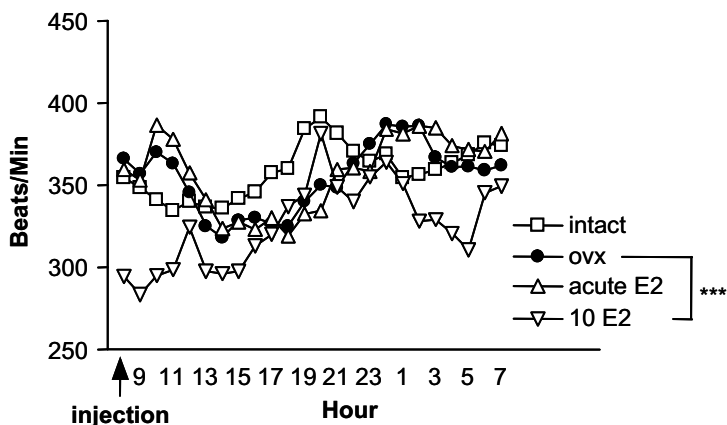


Figure 11. Heart rate for 24 hours in female normotensive WKY rats (a) and spontaneously hypertensive rats (SHR) (b), each rat acted as its own control. Intact, ovariectomy (ovx), 17β -estradiol treatment for one day (acute E2) and ten days (10 E2). Passive period (ranging from 12 - 17) and active period (the remaining of the day) based on pooled activity data. Arrow shows time for injection. *** $p < 0.001$. Data are expressed as means.

Fig. 11b SHR - Heart Rate



DISCUSSION

The present studies investigate the effect of oestrogen, the female sex hormone, on the cardiovascular system in normotensive and hypertensive subjects. We used both animal and human models to study the effects of oestrogen replacement on the central haemodynamic system and peripheral vascular reactivity. Because ethical limitations sometimes create difficulty in clarifying pathophysiological mechanisms in humans, animal models provide important complementary study designs. However, we do not always know whether the effect of a studied compound is applicable to both animals and humans.

In considering the effects of oestrogen treatment, we also must pay heed to the type of oestrogen used, its method of administration (first liver passage), short- vs long-term treatment and, for HRT, the possible additive effect of progesterone. In the present studies, 17 β -estradiol attenuated ambulatory BP and HR, but its effect on BP occurred only after short-term treatment in normotensive subjects. On the other hand, HRT did not influence BP or HR in hypertensive postmenopausal women, suggesting a selective effect of transdermal 17 β -estradiol or a counteracting effect of progesterone. HRT and 17 β -estradiol affected insignificantly the haemodynamic responses to stress, suggesting that oestrogen does not influence centrally regulated sympathetic outflow at maximal arousal. However, both 17 β -estradiol and HRT attenuated vascular adrenergic reactivity in hypertensive female rats and women. Applied on top of a precontracted rat resistance artery, 17 β -estradiol caused prompt relaxation, primarily through the release of NO. Conversely, oestrogen administered *in vivo* did not improve agonist-induced relaxation, although both short- and long-term 17 β -estradiol treatment and HRT reinforced muscarinic response in hypertensive female rats as well as postmenopausal normotensive and hypertensive women.

The acute effects of 17 β -estradiol on small mesenteric arteries *in vitro*

Previous reports on conduit and coronary vessels have documented a relaxing property of oestrogen [Salom 2002, Teoh 1999, Abou-Mohamed 2003]. *Paper I* confirms that applied 17 β -estradiol also promptly relaxes NA precontracted small resistance arteries from male and female rats, suggesting a non-genomic effect [Abou-Mohamed 2003, Salom 2002]; the classic nuclear receptor or gene transcription is probably not involved [Freay 1997]. Whether oestrogen releases NO [Collins 1994] and/or a hyperpolarizing agent from the endothelium (EDHF) or acts directly on VSMC, involving the potassium channel signalling system [Jiang 1991, Abou-Mohamed 2003], remains undetermined. Since L-NNA completely inhibited artery relaxation following administration of low dose 17 β -estradiol, the effect of oestrogen in small mesenteric arteries may depend mainly on NO. However, high concentrations of 17 β -estradiol elicited concomitant hyperpolarization of the female

arteries. Consequently, we confirm here the predominant NO release by low doses and an associated hyperpolarizing ability of high doses of 17 β -estradiol in the smaller arteries of female rats, effects previously reported in larger vessel types [Tep-areenan 2003, Collins 1994].

Despite a tendency towards increased sensitivity in male small mesenteric arteries, the sensitivity and maximal response to applied 17 β -estradiol did not differ significantly between genders. Previous studies investigating gender differences and differences in effect that result from pre-experimental status of sex hormones in females are inconsistent. Some reported higher sensitivity to acutely administered 17 β -estradiol in male mesenteric arteries compared to females [Tep-areenan 2003] or diestrous (low oestrogen) females [Shaw 2001]. Others suggested that testosterone intensifies the acute effects of oestrogen, increasing our understanding of why arteries from proestrous females (high levels of oestrogen and testosterone) might respond better than those from diestrous females [Shaw 2001]. Interestingly, in larger atherogenic arteries a greater sensitivity to oestrogen in females is present, if a difference has been reported [Lamping and Nuno 1996, Freay 1997, le Tran 1997].

Incubation with 17 β -estradiol did not affect contractile responses to either NA, 5-HT or potassium (*Paper I*), thus emphasising the importance of specifying the method when referring to “acute effects” of oestrogen. Furthermore, these results do not support previous reports on larger vessel types, where an attenuating effect on the 5-HT-induced contraction in human internal mammary arteries and basilar arteries from rabbits indicated a selective effect of oestrogen on 5-HT receptors [Mugge 1997, Shay 1994].

The effects of oestrogen on endothelial function

Our finding that 17 β -estradiol confers direct relaxation in small resistance arteries raised the question whether oestrogen administered *in vivo* might improve endothelial function, especially in hypertensive subjects with expected impaired relaxation. Evidence of improved endothelial function has been shown *in vivo* by increased flow mediated and agonist induced vasodilation in the brachial artery after oestrogen treatment [Saitta 2001, Lima 2005]. An up regulated consecutive- and agonist-induced NO release by oestrogen occur in animals [Huang 1997] and in subcutaneous arteries from healthy postmenopausal women after incubation with 17 β -estradiol [Kublickiene 2005].

In the present studies, neither transdermal 17 β -estradiol treatment (24 h) nor HRT (six mo) improved maximal relaxation capacity in subcutaneous arteries of normotensive and hypertensive postmenopausal women, respectively (*Papers IV* and *V*). Nevertheless, we determined that oestrogen modulates ACh-induced relaxation in such arteries and also in mesenteric arteries from ovx-SHR treated with 17 β -estradiol (*Paper II*). However, relaxation to SP remained unaffected (*Papers IV* and *V*), suggesting selective modulation of the muscarinic response.

Because relaxation was poorly inhibited by L-NNA (*Papers IV and V*) and not affected by indomethacin, we suggest that ACh causes non-NO-, non-prostanoid-dependent relaxation in human subcutaneous arteries (*Paper IV*). Previous reports show that ACh poorly activates the NO/L-arginine pathway in such vessels [Buus 2000]. We further demonstrate that subcutaneous arteries release NO via receptor stimulation by other agonists, e.g., SP. However, ACh released NO to a small extent in the hypertensive placebo group, but not after HRT (*Paper V*) or in normotensive women (*Paper IV*). A gender dependent, altered balance in the release of endothelial derived factors in response to muscarinic receptor stimulation has previously been shown in mesenteric arteries from male and female rats [McCulloch and Randall 1998]. In accord with our observations in hypertensive older women, the relaxation capacity to ACh did not differ in mesenteric arteries from oestrogen-deprived older female rats compared to intact and oestrogen-treated rats, although the proportion of NO and EDHF released from the endothelium varied [Nawate 2005]. Due to increased eNOS activity and induction, NO-mediated relaxation increased after ovariectomy, and EDHF-mediated relaxation declined due to decreased myo-endothelial gap junctions [Nawate 2005].

In our model, oestrogen did not affect the agonist induced or consecutive NO release, since relaxation to SP and contractile response to NA remained unaffected (*Papers IV and II*). However, we observed reduced sensitivity to NA after HRT (six mo) (*Paper V*), possibly due to stimulated consecutive NO production or more likely a direct effect on the alpha-adrenoceptors (see below). Since oestrogen does not affect relaxation to sodium nitroprusside, a direct activator of guanyl cyclase in VSMC that increases the intracellular level of cGMP, many studies have excluded the possibility that oestrogen might alter VSMC reactivity to NO [Darblade 2002, Huang 1997].

The reinforced ACh-induced relaxation by 17 β -estradiol and HRT more likely results from increased release of EDHF. A small depolarisation with KCl attenuated relaxation to ACh in placebo-treated vessels (*Paper IV*), suggesting participation of EDHF in these small arteries. This was further evaluated in *Paper V* where we more specifically inhibited the EDHF pathway by inhibition of the conductance Ca²⁺-sensitive K- channels [Chen and Cheung 1997, Petersson 1997]. The sensitivity to ACh decreased following EDHF inhibition, especially in the placebo group (*Paper V*). Thus, treatment with 17 β -estradiol or HRT counteracted the inhibition of the EDHF pathway and reinforced the response to muscarinic stimulation compared to placebo, suggesting a specific influence on the ACh signalling pathway.

17 β -estradiol-treatment in SHR also affected ACh-induced relaxation, suggesting a general effect of oestrogen on the muscarinic response independent of strain and type of oestrogen. The paradoxical vasoconstriction to high doses of ACh observed in intact and ovx-SHR decreased after one and ten days of 17 β -estradiol treatment. Others have reported vasoconstriction to high doses of ACh in male SHR [Watt and Thurston 1989]. Since low

concentrations of ACh relax precontracted vessels to the same magnitude in normo- and hypertensive subjects (*Paper II*), reduced relaxation following high doses of ACh likely represents a functional effect. Indeed, the paradoxical vasoconstriction to higher doses of ACh occurs prior to structural changes in the vascular wall [Watt and Thurston 1989]. Increased production and release of a cyclooxygenase-dependent contracting factor, probably the prostaglandin thromboxane A₂ [Watt and Thurston 1989] or superoxide [Fu-Xiang 1992], may explain ACh-induced vasoconstriction. In accordance with our results (*Paper II*), oestrogen counteracts this paradoxical phenomenon in skeletal muscle arterioles from female SHR [Huang 1997]. Following exposure to oestrogen, the release of basal- and agonist-induced NO from the endothelium increases, preventing or even overcoming the contractile effect of thromboxane A₂ [Huang 1997]. However, the mesenteric arteries of intact fertile female SHR studied here also responded with vasoconstriction, and the counteraction by exogenously-administered 17 β -estradiol suggests interference by other ovarian hormones or a pharmacological effect due to high doses of 17 β -estradiol. In a study on ovx mongrel dogs, coronary arteries showed higher sensitivity to ACh in dogs treated with oestrogen compared to untreated dogs as well as those treated with oestrogen plus progesterone [Miller and Vanhouette 1991]. Thus, progesterone might counteract oestrogen's beneficial effects on endothelial function, which might be the case in ovulating females.

Oestrogen's ability to modulate some but not all endothelium-dependent responses emphasises that several mechanisms likely participate in endothelial factor release. We determined a reinforced ACh induced relaxation following 17 β -estradiol and HRT treatment of subcutaneous arteries from postmenopausal normotensive and hypertensive women and also of mesenteric arteries from hypertensive rats, suggesting that the effect of oestrogen occurs independent of strain and type. However, the muscarinic response in normotensive WKY rats remained unaffected by such treatment, possibly due to relatively young age and minimal deprivation of oestrogen in normotensive rats (*Paper II*) compared to old normotensive women (*Paper IV*). Morphological disturbances and endothelial cell layer disruption occur in subcutaneous arteries even from healthy normotensive postmenopausal women [Kublickiene 2005].

The effect of oestrogen on the ACh and muscarinic receptor responses has been studied in other tissues. Oestrogen treatment results in reduced contraction to ACh in the urine bladder [Yildiz 2005], probably due to a down-regulation of M2 receptors [Liang 2005]. On the other hand, oestrogen enhances sensitivity to ACh in the uterus [Abdalla 2000], due either to increased density of muscarinic receptors [Batra 1990] or modulation of the intracellular signalling pathways [Abdalla 2000]. Different types of muscarinic receptors localized in the vascular wall [Phillips 2000] might provide important sites of action for oestrogen, modulating the endothelium's capacity to release vasodilating and perhaps vasoconstricting agents. The release of endothe-

lium-derived relaxing factors by muscarinic activation is partially coupled to a pertussis toxin-sensitive regulatory protein in endothelial cells [Miller and Vanhouette 1991]. Oestrogen affects the pertussis-sensitive cAMP signal transduction system [Miller and Vanhouette 1991], possibly explaining the variability of oestrogen's effects on ACh-induced relaxation in different vessel types [Miller and Vanhouette 1991]. Another possible mechanism involves the EDHF pathway where oestrogen enhances connexin 40 and 43, both known participants in the formation of gap junctions that transfer the EDHF signal from the endothelium to VSMC [Nawate 2005].

In summary, the effect of oestrogen on the endothelium, likely coupled to the muscarinic response, might result from increased density or sensitivity of the receptor *per se* and/or an effect on the intracellular signalling system involving the EDHF pathway. If oestrogen reinforces EDHF response, it likely affects other agonist-induced relaxation using this pathway as well. Such effects will require further evaluation.

The effects of oestrogen on vascular adrenergic reactivity

Vascular adrenergic reactivity is an important factor in hypertension [Esler 1989]. Previous studies have shown increased vascular neurogenic response in small arteries from male SHR compared to normotensive controls [Nilsson and Folkow 1982, Nilsson and Sjoblom 1984], possibly resulting from increased NA synthesis [Collis 1980]; increased neuronal release of NA in response to nerve stimulation [Tsuda and Masuyama 1990]; inhibited neuronal re-uptake; or increased VSMC reactivity to NA [Mulvany 1980] in hypertensive subjects. We show here that vascular neurogenic response in mesenteric arteries from intact female SHRs did not differ compared to normotensive controls (*Paper II*). Following ovariectomy, however, contractile response to nerve stimulation increased in SHR, mimicking the pattern seen in males. Exogenously administered 17 β -estradiol diminished the difference after one day of treatment, suggesting that the ovarian hormone oestrogen causes a rapid attenuated response, possibly due to a non-genomic mechanism. Similarly in *Paper V*, HRT (six mo) attenuated contractile response to TNS in subcutaneous arteries from hypertensive postmenopausal women.

Due to the presence of cocaine and propranolol during our experiments, the effect of oestrogen (see above) cannot result from either altered NA re-uptake or an effect on beta-adrenergic receptors [Ferrer 1996], which counteracts the vasoconstrictor effects of NA. With TNS, we electrically stimulated all neuromuscular junctions around the vessels. In addition to sympathetic nerve fibres, the adventitia of intestinal arteries contains cholinergic nerve fibres, which release a muscarinic receptor agonist that causes vasodilation [Andriantsitohaina and Surprenant 1992]. Therefore, an affected muscarinic response in small mesenteric arteries in the presence of oestrogen (see above) theoretically could account for the attenuated response to TNS. However, the complete reversion by α_1 -receptor blockade, together with the unaffected response to exogenously applied NA by ovariectomy, sug-

gests that increased contractile response is adrenergic, although not caused by postjunctional modifications at the adrenergic receptor level (*Paper II*). Compared to normotensive controls, NA release per impulse is increased in resistance arteries of male SHR, either on a quantum basis or due to denser innervation [Starke 1977]. Moreover, increased concentration of angiotensin II facilitates NA neurotransmission in male SHR [Faria and Salgado 1992], and greater overflow of endogenous NA in male SHR links with altered calcium handling during neurotransmission in male SHR compared to WKY [Tsuda and Masuyama 1990]. Based on the rapid onset of the effect of oestrogen, a non-genomic calcium blocking mechanism may explain reduced neurotransmission in females with hypertension [Jiang 1992].

Hypertensive postmenopausal women treated with HRT experienced concomitantly reduced sensitivity to exogenously applied NA (*Paper V*) as well as attenuated response to TNS. Although these observations point towards reduced sensitivity at the post-junctional adrenergic receptor level, we can not rule out simultaneously reduced neurotransmission in accordance with the findings in female SHR (*Paper II*). Unfortunately, scope of our work did not allow us to consider the effect of 17β -estradiol on the contractile response to TNS in normotensive women (*Paper IV*); thus, we can only speculate whether the observed differences in hypertensive rats and women depend on the strain or the type of oestrogen used, or result from progesterone.

A previous study on perimenopausal women showed reduced vasoconstrictor response to NE *in vivo* together with reduced total body spill over of NE following oestrogen supplementation (oral estradiol valerate), indicating an attenuated adrenergic receptor response together with reduced sympathetic neural activity [Sudhir 1997]. Because short-term 17β -estradiol exposure did not affect contractile response to NA in normotensive women (*Paper IV*), the reduced vasoconstrictor response to NA might be time-dependent. More likely, reduced adrenoceptor responsiveness in hypertensive women (*Paper V*) depends on oestrogen type [Sudhir 1997] or added progesterone [Orosz 1983]. Increased uterine blood flow in the follicular phase, when the oestrogen/progesterone ratio is high, depends on decreased numbers of alpha-adrenoceptors and/or modulation of the intracellular signalling pathway [Ford 1984]. However, the density of α_1 -receptors was not influenced in mesenteric arteries by the estrous cycle in the referred study [Ford 1984] and consequently the difference in post-junctional adrenergic sensitivity after oestrogen exposure (*Paper II* and *V*) might depend on the different vascular beds studied. Progesterone receptors have also been identified in the vascular wall [Ingegno 1988], but their role has received less attention than oestrogen receptors. Another interesting hypothesis might involve the effect of progesterone on adrenergic receptors, while oestrogen affects the outflow of adrenergic agonists from periarterial sympathetic nerves. We observed no reduction in sensitivity to NA in intact SHR (*Paper II*). However, we did not consider the oestrous cycle, which may influence results.

The effect of oestrogen treatment on blood pressure in normotensive and hypertensive subjects

Surprisingly, ovariectomy resulted in decreased MAP in normotensive rats (*Paper III*). It could be due to a time effect, but SHR ran in a parallel manner and were unaffected by ovx. Otherwise, ovariectomy or menopause do not affect [Doursout and Chelly 2002] or increase BP [Amigoni 2000, Portaluppi 1997], especially in previously-hypertensive subjects [Clark 2004, Hinojosa-Laborde 2004].

Oestrogen treatment (17 β -stradiol) (24 h) significantly reduced ambulatory BP in normotensive postmenopausal women (*Paper IV*) and also in hypertensive and normotensive ovx rats (*Paper III*). Ten days of 17 β -estradiol treatment increased MAP to a small extent in normotensive ovx rats (*Paper III*) but substantially attenuated MAP in ovx-SHR (*Paper III*), previously shown in intact normotensive and hypertensive rats treated with oestradiol [Stonier 1992]. A longer oestrogen regimen (HRT) did not affect ambulatory BP in hypertensive women possibly because we, for ethical reasons, aimed for normalized office BP (*Paper V*). Consequently, subjects were treated with one or several antihypertensive drugs during the 12-month period, that might conceal a potential pressure lowering effect by oestrogen *per se*. Another explanation might include the oestrogen type or a counteracting effect by progesterone (*Papers III and IV*).

The route of administration seems particularly important in reports about a BP-lowering effect of oestrogen in humans. A study on normotensive postmenopausal women reported lower SBP following treatment with transdermal oestrogen (17 β -estradiol) and smaller effects following orally-administered HRT [Zacharieva 2002]. Additionally, this difference occurs in both normotensive and hypertensive postmenopausal women [Mueck and Seeger 2004]. Therefore, we conclude oestrogen's ability to lower BP is modest in postmenopausal subjects and more likely occurs with transdermally or subcutaneously administered 17 β -estradiol. Moreover, oestrogen's effect on BP is greater in previous hypertensives compared to normotensives [Jespersen 1983, Stonier 1992].

The effect of oestrogen on heart rate in normotensive and hypertensive subjects

Few reports have investigated the effect of oestrogen on HR. We observed a marked decrease in HR in both normo- and hypertensive rats after ten days of 17 β -estradiol treatment (*Paper III*) as well as attenuated daytime HR in normotensive postmenopausal women after only 24 h of 17 β -estradiol exposure (*Paper IV*). HRT did not influence HR in hypertensive women (*Paper V*), which might depend on the use of beta blockers, type of oestrogen, or interference with progesterone. An earlier report postulated counteracting effect of progesterone in postmenopausal women [Zacharieva 2002], on the

other hand, 17β -estradiol reduced HR after acute intravenous injection [He 1998] and after longer treatments in normotensive and hypertensive postmenopausal women [Luotola 1983]. The present data confirm that this effect, also seen in the rat model, likely does not depend on a subject's BP or HR prior to treatment with oestrogen. Oestrogen's effect on HR might rely on increased vagal and reduced sympathetic tone [Mercurio 2000, Liu 2003].

The effect of oestrogen treatment on the autonomic nervous balance

HRV analysis provides better method than HR for studying vagal and sympathetic regulation of the heart. This method allows analysis of ANS regulation of the heart non-invasively by evaluating the interval oscillations between consecutive heart beats as well as oscillations between consecutive instantaneous HR [Task Force of the European Society of Cardiology 1996]. When analysing a 24-hour HRV recording, several parameters are included, and the time and frequency domain variables are strongly correlated with each other due to both mathematical and physiological relationships [Task Force of the European Society of Cardiology 1996]. However, in order to compare our results with other reports, the current study includes both time and frequency domain variables.

Several reports on the effects of oestrogen on HRV have shown a higher HF [Frag 2002] and lower LF/HF ratio [Liu 2003, Yildirim 2001] indicating increased parasympathetic tone on the sinus node by the female sex hormone. This effect does not seem to depend on type of oestrogen used; both oestrogen replacement therapy (ERT) and HRT [Liu 2003, Yildirim 2001] to postmenopausal women have shown documented effects. Furthermore, endogenous oestrogen also seems protective, i.e., premenopausal women have higher HF compared with postmenopausal women and men [Liu 2003].

Although most studies seem to report a positive effect of oestrogen on the autonomic modulation of the heart, there are some inconsistencies. In an observational study, HRT was associated with lower HR and higher HRV, but adjustments for age and risk factors for coronary heart disease illuminated these differences [Carnethon 2003]. In accordance with our study on hypertensive otherwise healthy postmenopausal women HRT in normotensives showed no effect on HRV after three or six months [Fernandes 2005, Niskanen 2002]. Small sample size and the use of antihypertensive drugs in the current study might influence the results.

We observed a small effect on DBP response to stress following 17β -estradiol exposure (24 h) in normotensive women (*Paper IV*), concurring with another study on healthy postmenopausal women treated with 17β -estradiol for three weeks [Ceresini 2000]. However, these results were not repeated after long-term HRT in hypertensive postmenopausal women (*Paper V*) or in the rat model given 17β -estradiol (*Paper III*). Few studies have investi-

gated estrogen's effect on cardiovascular response to stress in animals, perhaps due to technical difficulties in measuring BP and HR in freely moving animals. Concurrent with our data, no effect of 17β -estradiol treatment after seven days in ovx female SHR exposed to open field stress was reported [Eikelis and Van Den Buuse 2000]. However, 17β -estradiol combined with progesterone resulted in significantly lower BP at stress [Eikelis and Van Den Buuse 2000], data not supported by our study in hypertensive postmenopausal women treated with oral HRT (*Paper V*). Therefore, the discrepancy in our results on the effects of oestrogen on sympathetic outflow might depend on species as well as treatment regimen.

CONCLUDING REMARKS

Our present studies conclude that oestrogen attenuates adrenergic reactivity in small resistance arteries from ovx hypertensive rats and postmenopausal women. A reduced adrenoceptor vasoconstriction in resistance arteries might contribute to improved local blood flow to the peripheral tissues despite maintained BP. Oestrogen's effect on BP may depend on type of oestrogen used together with the pre-existing pressure of the subject. Attenuated BP after 24 hours of 17β -estradiol treatment occurred in normotensive- and hypertensive subjects, an effect which only sustained in hypertensive rats with a longer treatment period. Further, acute application of 17β -estradiol *in vitro* released NO from the endothelium and relaxed precontracted resistance arteries. Oestrogen administered *in vivo* modulated the muscarinic response selectively in arteries from postmenopausal women and hypertensive rats, independent of oestrogen type and exposure time, but did not influence the relaxation capacity *per se*.

Our findings suggest that oestrogen protects the vascular tone of resistance arteries in hypertensive but otherwise healthy postmenopausal women. The effects of oestrogen on cardiovascular outcomes in this selective group of subjects require further evaluation in larger studies. Experimental studies seeking to evaluate the site of action for a specific substance are much more easily accomplished in animal models. *Papers I–V*, presented here, strongly suggest that distinguishing between the types of oestrogen used has greater value than distinguishing between species, thus supporting the need for further investigations with identical treatment regimens regarding oestrogen type, progesterone supplementation, and exposure time in both animals and humans before we know why most experimental studies find a protective effect of oestrogen on the vascular tree and larger clinical trials do not confirm the protective role of HRT on cardiovascular health.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Det kvinnliga könshormonet östrogen har visat sig vara viktigt inte bara för kvinnlig könsmognad utan även för skelettet, hjärnan och hjärtkärlsystemet. Efter menopaus sjunker östrogennivåerna hos kvinnor vilket kan ge symtom så som värmevallningar, torra slemhinnor, urinvägsinfektioner, yrsel och hjärtklappning. Hormonersättning i övergångsåldern lindrar dessa symtom men det finns också en ökad risk för blodproppsbildning och bröstcancer. Epidemiologiska studier (befolkningsstudier som värderar hur olika faktorer inverkar på hälsan) har visat att östrogensubstitution till postmenopausala kvinnor verkar skydda mot insjuknandet i hjärtkärlsjukdomar som stroke, kärlkramp och hjärtinfarkt. När man undersökt detta vidare med experimentella studier har man funnit att östrogen förbättrar blodfetsprofilen, minskar åderförkalkning i hjärtats kranskärl, ökar blodflödet till vissa organ och påverkar blodtrycket.

Under början av 2000 talet visade dock två större kliniska behandlingsstudier att hormonersättning (östrogen plus progesteron) inte minskade risken för insjuknandet i stroke och hjärtinfarkt, vare sig hos friska eller hjärtkärlsjuka postmenopausala kvinnor. Med tanke på de positiva effekter man ändå ser med östrogen i epidemiologiska och mindre experimentella studier kan man fundera på varför man inte fann någon skyddande effekt i de större kliniska studierna. Skillnader i resultat kan bero på vilken typ av östrogen som använts, skillnader i progesterontillägg (progesteron ges som skydd mot livmodercancer om kvinnan har kvar sin livmoder), vid vilken tidpunkt man påbörjar behandling (under eller efter klimakteriet) och om kvinnorna har andra sjukdomar där östrogen kan påverka.

Denna avhandling studerar hur östrogen, givet under kortare och längre tid, påverkar blodtryck och hjärtfrekvens samt funktionen hos de små blodkärl som är viktiga för reglering av blodtrycket (= resistenskärl). Östrogen gavs till postmenopausala kvinnor och honråttor (där äggstockarna tagits bort) med normalt och högt blodtryck.

När vi gav östrogen i form av 17β -estradiol via huden till kvinnor med normalt blodtryck och råttor med både normalt och högt blodtryck sänktes blodtrycket inom 24 timmar men detta var endast bestående hos råttor som hade högt blodtryck från början. Även hjärtfrekvensen sjönk efter 17β -estradiol behandling men vi såg ingen större effekt på blodtryck eller hjärtfrekvens under stress. Detta kan tala för att östrogen påverkar det parasympatiska nervsystemet som dominerar i vila men har små effekter på det sympatiska nervsystemet som slås på under stress. En högre aktivitet av det parasympatiska nervsystemet och en lägre aktivitet av det sympatiska nervsystemet har visat sig skydda mot t ex hjärtinfarkt och våra studier skulle således tala för en skyddande effekt av just denna östrogen typ (17β -estradiol). Hos de kvinnor som hade ett högt blodtryck från början såg vi däremot ingen effekt

på blodtryck eller hjärtfrekvens efter en längre tids behandling med konjugerat östrogen plus progesteron givet i tablett form. Vi kunde heller inte med denna behandling bekräfta någon ökad parasympatisk aktivitet efter att ha undersökt balansen mellan det sympatiska och parasympatiska nervsystemet under dygnet med så kallad hjärtfrekvensanalys.

Att resultaten skilde sig mellan råttor med högt blodtryck, kvinnor med normalt blodtryck och kvinnor med högt blodtryck kan bero på typen av östrogen som användes eller tillägget med progesteron. Det kan också bero på behandlingstidens längd och på att kvinnorna med högt blodtryck var behandlade med blodtryckssänkande medicin. Trots ett oförändrat blodtryck fann vi hos dessa kvinnor en lägre känslighet för stresshormonet noradrenalin i de små blodkärlen efter hormonersättning, vilket talar för att östrogen förbättrar blodflödet till vävnaderna. Hos råttor med högt blodtryck sågs en lägre noradrenalinfrisättning i de små resistenskärlen, vilket också minskar kärlsammandragningen och gynnar perifert blodflöde. Båda typerna av östrogen (17 β -estradiol och konjugerat östrogen plus progesteron i tablettform) verkar således ha en skyddande effekt på kärlnivå hos dem som har ett högt blodtryck.

Östrogen påverkade också frisättningen av olika faktorer från de celler som bekläder insidan av blodkärlen (= endotelet). Dessa faktorer gör att muskelcellerna som finns i kärlväggen slappnar av och gynnar blodgenomströmningen. När östrogen tillsattes i organbad sågs en direkt kärlvidgande effekt på små artärer som stimulerats med stresshormonet noradrenalin, men när råttor och kvinnor fick östrogenbehandling, via huden eller i tablettform, förbättrades inte blodkärlets endotelberoende förmåga att slappna av. Dock påverkades balansen mellan de faktorer som frisattes från endotelet. Vilken betydelse detta har vet vi inte i nuläget och för att ta reda på det krävs fortsatta studier.

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