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Vasoactive biomarkers and oxidative stress in healthy recently postmenopausal women treated with hormone replacement therapy

S. Maffei, A. Mercuri, C. Prontera, G. C. Zucchelli and C. Vassalle

CNR Institute of Clinical Physiology, Pisa, Italy

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ABSTRACT

Background Despite biologically plausible mechanisms for cardiac protection from estrogen therapy, recent clinical trials have suggested possible cardiovascular risk rather than benefit. However, it has been speculated that cardioprotective benefits from hormone replacement therapy (HRT) may be more evident in the early postmenopausal period. We have previously reported early beneficial effects on biochemical markers of endothelial function in healthy women after short-term estradiol replacement therapy. In this study we aimed to evaluate the effect of long-term HRT on different vasoactive factors and oxidative stress in healthy recently postmenopausal women.

Methods Fifteen women (age 50 ± 1 years, time since menopause 1.6 ± 0.1 years) were randomized to a sequential oral and transdermal estradiol regimen (2 mg oral micronized 17β-estradiol/day or 1.5 mg 17β-estradiol gel/day). Oral dydrogesterone (10 mg/day, 12 days/month) was then cyclically combined with either of the estrogen therapies for 1 year. Blood samples were collected at baseline and after 1, 2, 6 and 12 months of therapy to evaluate levels of follicle stimulating hormone (FSH), estradiol, 6-keto $PGF_{1\alpha}$ (prostacyclin metabolite), nitrite/nitrate, epinephrine, norepinephrine, 8-isoprostane (8-epi $PGF_{2\alpha}$) and lipid profile values.

Results FSH levels decreased ($p < 0.001$) while estradiol levels increased ($p < 0.001$) during HRT. Levels of epinephrine ($p < 0.001$), norepinephrine ($p < 0.01$), mean blood pressure ($p < 0.01$) and low density lipoprotein (LDL) cholesterol ($p < 0.01$) decreased, and nitrite/nitrate levels increased ($p < 0.01$) during HRT, which did not significantly affect 8-epi $PGF_{2\alpha}$ levels.

Conclusions One-year HRT significantly reduced the levels of catecholamines, mean blood pressure and LDL cholesterol while it increased levels of nitrite/nitrate, indicating cardiovascular benefit in healthy recent postmenopausal women. Levels of 8-epi $PGF_{2\alpha}$ did not change, suggesting no evident relationship between HRT and oxidative stress.

Correspondence: Dr C. Vassalle, Istituto di Fisiologia Clinica, Area di ricerca CNR, Via Moruzzi 1, I-56100, Pisa, Italy

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INTRODUCTION

Although it is accepted that estrogen exerts a protective role on the cardiovascular system during fertile life, and that the prevalence of cardiovascular disease increases after the menopause, results obtained from recent randomized clinical trials have suggested that hormone replacement therapy (HRT) may even increase the risk of cardiovascular events[1–4]. These findings have discouraged the use of HRT in postmenopausal women, although many flaws have been identified, including the lack of menopausal symptoms, older age of women enrolled and the initiation of estrogen late after menopause[5]. In particular, the idea is growing that the maintenance of a healthy endothelium after menopause is important to retain an effective response to estrogen administration and that maximal vascular benefit from the HRT regimens require early treatment, near the onset of menopause[5,6]. Accordingly, we have recently shown that the use of short-term estrogen replacement therapy induces early beneficial changes on some biochemical markers of vascular function in women in recent postmenopause[7]. However, whether these favorable and important vascular effects might be retained by long-term estrogen treatment when combined with a progestogen remains to be determined.

Thus, in this study we aimed to evaluate the effect of 1-year HRT treatment on different vasoactive factors. To address this purpose, nitrite/nitrate (NOx, final and stable metabolites of the nitric oxide metabolic pathway), epinephrine and norepinephrine, 6-keto $PGF_{1\alpha}$ (as index of prostacyclin), lipid profile values together with levels of 8-isoprostane, a marker of lipid peroxidation and oxidative stress, were monitored during the study in a group of healthy women who were recently postmenopause.

MATERIALS AND METHODS

Enrolment of patients was conducted according to criteria previously described[7,8]. In brief, the study included 15 healthy postmenopausal women (age 50 $\pm$ 0.7 years, mean $\pm$ standard error; time since menopause 1.6 $\pm$ 0.1 years), randomized to start oral (2 mg oral micronized 17β-estradiol daily) or transdermal estradiol therapy (1.5 mg 17β-estradiol gel/daily) for 1 month. Subsequently, the treatment was crossed over for the 2nd month of therapy. Then, after taking preference into account, patients continued the treatment with oral ($n = 5$) or transdermal ($n = 10$) treatment plus oral dydrogesterone for 12 days/month (Figure 1).

Menopausal status was confirmed by serum concentrations of follicle stimulating hormone (FSH) >40 mIU/ml and estradiol <20 pg/ml. Subjects enrolled in the study had similar lifestyles

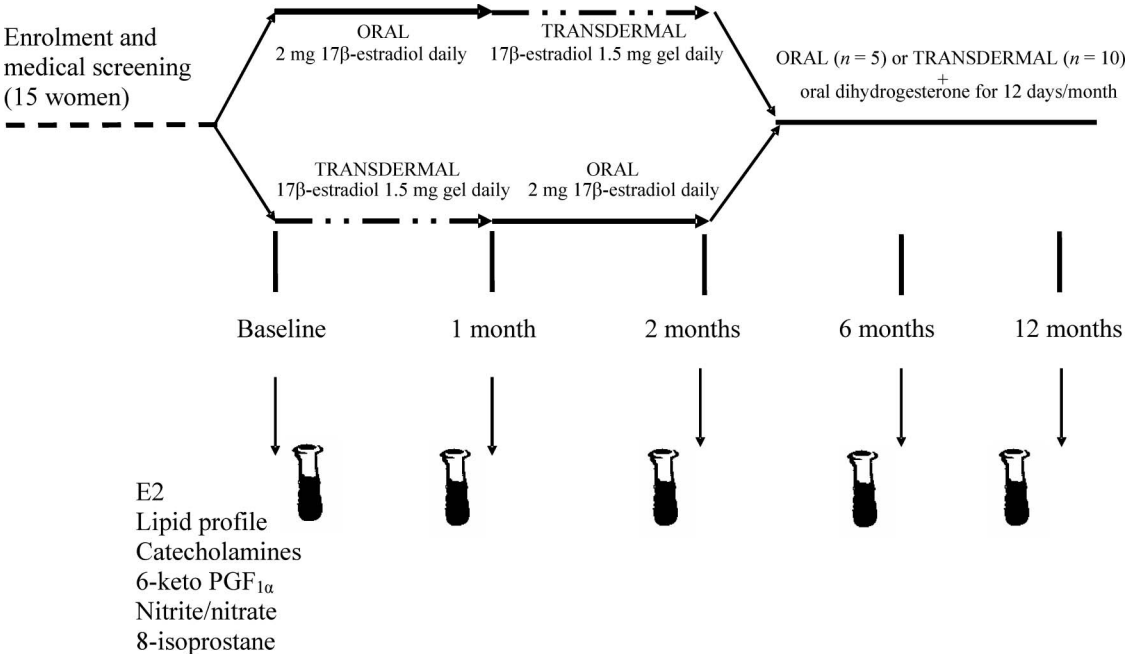

Figure 1 Flow chart describing women's progression through different phases of the study protocol

and dietary habits that were characteristic of the Mediterranean diet; all were requested to avoid any changes in dietary habits during the investigation.

None of the women had risk factors for coronary artery disease (cholesterol >220 mg/dl, diabetes, hypertension, cigarette smoking or family history). None had ever received HRT, or had taken any medication known to affect blood pressure within the last 6 months. Fully informed consent was obtained from each subject entering the study, and the experimental protocol was approved by the local Hospital Ethics Commitee.

Blood samples were collected in the morning after overnight fasting at baseline, and after 1, 2, 6 and 12 months (Figure 1). Blood pressure was also measured at each visit, and the mean was recorded of three measurements taken at 5-min intervals by means of a conventional sphygmomanometer with the patient in the supine position. All blood samples were centrifuged immediately in a refrigerated centrifuge at 2000 rpm for 15 min and samples frozen at -80°C until assayed.

Samples for FSH and estradiol were analyzed by fully automated laboratory methods (Access, Beckman). Plasma concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were determined by standard laboratory methods. The concentration of low density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. Plasma epinephrine and norepinephrine levels were measured by an automated HPLC analyzer based on fluorescence detector (HLC-725, Eurogenetics-Italia, Turin, Italy).

Samples for NOx assay were previously ultrafiltered through 30 kDa molecular weight cut-off filters and centrifuged at 1000 *g* for 60 min, then assayed by a colorimetric assay kit based on Griess reaction (Cayman, Ann Arbor, USA).

Concentrations of 6-keto prostaglandin $F_{1\alpha}$ and 8-isoprostane were determined by a kit for competitive enzyme-linked immunoassay (Cayman Chemicals, Ann Arbor, MI, USA). Before the assay procedures, samples were subjected to purification by means of Supelclean LC-18 columns (Supelco, Bellefonte, PA, USA), according to manufacturer's instructions.

Statistical analysis

Data are expressed as mean $\pm$ standard error. Statistical analysis performed included simple regression analysis, repeated-measure ANOVA analysis, and Scheffe's test using the statistical package Statview, version 5.0.1 (SAS Institute, Abacus Concept, Inc., Berkeley, California). p levels lower than 0.05 were considered statistically significant.

RESULTS

Demographic and clinical characteristics of women are detailed in Table 1. Each woman had been amenorrheic for at least 1 year, and all presented with climacteric symptoms and requesting HRT. The FSH and estradiol baseline plasma levels were in agreement with their postmenopausal state – 108 ± 11 mIU/ml and 19.3 ± 1 pg/ml, respectively. The FSH concentration significantly decreased (47 ± 7, 69 ± 8, 59 ± 7 and 51 ± 7 mIU/ml for 1 month and 3, 6, 12 months, respectively, $p < 0.001$) while estradiol plasma levels significantly increased (165 ± 22, 152 ± 25, 145 ± 21 and 159 ± 24 pg/ml; $p < 0.001$) during HRT treatment, as expected (Figure 2).

Epinephrine levels (from 30 ± 7 to 13 ± 2, 13 ± 2, 16 ± 2 and 14 ± 2 pg/ml; $p < 0.001$) and norepinephrine levels (from 439 ± 52, to 372 ± 57, 345 ± 43, 289 ± 31 and 295 ± 37 pg/ml; $p < 0.01$) significantly decreased during the treatment (Figure 3). Mean blood pressure showed a significant reduction during the study (from 92 ± 2 to 91 ± 2, 87 ± 2, 86 ± 2 and 85 ± 2; $p < 0.01$) (Figure 4).

A significant reduction in LDL levels ($p < 0.01$) was also observed, while NOx values ($p < 0.01$) significantly increased (Table 2). Also 6-keto $PGF_{1\alpha}$ levels increased during HRT, although the increases did not reach statistical significance (Table 2).

A positive correlation was observed between 8-isoprostane and body mass index ($p < 0.01$, $r = 0.72$) and age ($p = 0.07$, $r = 0.5$) in our population (Figure 5). However, HRT did not significantly modify the concentration of 8-isoprostane (Table 2).

Table 1 Demographic and physiological parameters observed in 15 healthy recently postmenopausal women admitted to start hormone replacement therapy

Age (years)	50 ± 0.7
Body weight (kg)	61 ± 2.7
Body length (cm)	159 ± 1.4
Body mass index (kg/m^2)	23 ± 0.8
Age at menopause (years)	49 ± 0.6
Years since menopause	1.6 ± 0.1

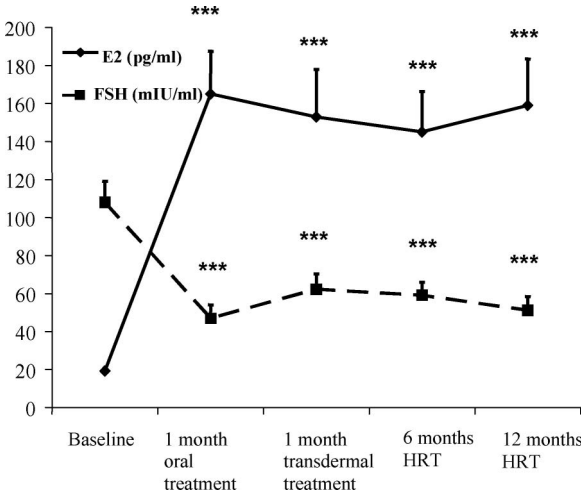

Figure 2 Changes in estradiol (E2) and follicle stimulating hormone (FSH) levels before and during 1-year hormone replacement therapy (HRT) in 15 healthy women in recent menopause. $***p < 0.001$ vs. baseline

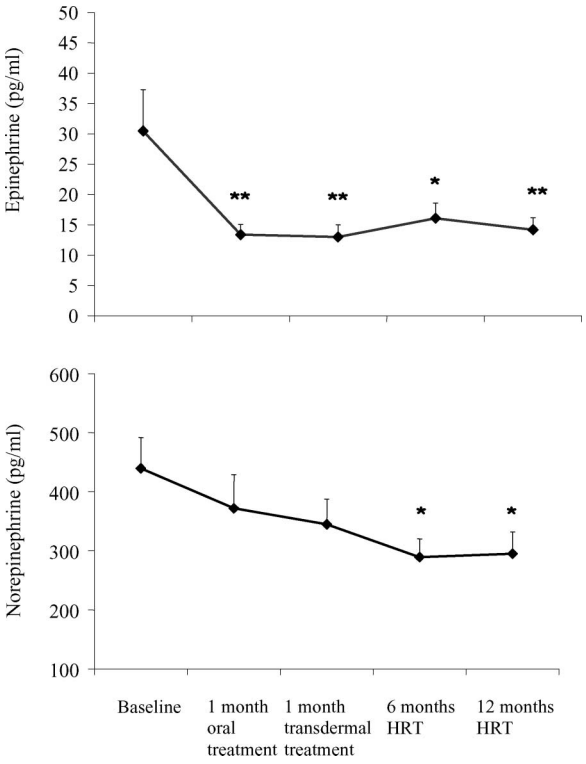

Figure 3 Changes in epinephrine and norepinephrine concentrations before and during 1-year hormone replacement therapy (HRT) in 15 healthy women in recent menopause. $*p < 0.05$; $**p < 0.01$ vs. baseline

DISCUSSION

In a previous study, we have reported vasoprotective changes after short-term replacement

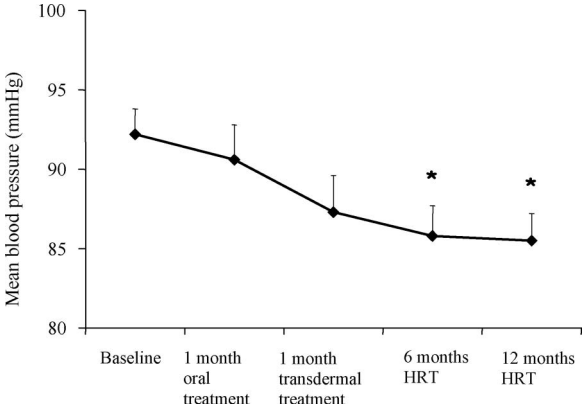

Figure 4 Changes in mean blood pressure levels before and during 1-year hormone replacement therapy (HRT) in 15 healthy women in recent menopause. $*p < 0.05$ vs. baseline

estrogen therapy, in the younger category of postmenopausal women[7]. However, it remained to verify the consequences of longer treatment, and whether the addition of a progestin could attenuate some protective effects of estrogen. In this study, we observed that, in healthy recently postmenopausal women, HRT for 1 year induces and maintains significantly favorable changes in physiological and biochemical markers of vascular function, without particular significant repercussion on the oxidative stress status.

In particular, HRT significantly lowered levels of epinephrine, norepinephrine and mean blood pressure, suggesting improvement of cardiovascular compliance. Hypertension is more common in men than women in younger age. However, this tendency is lost with aging, and women after menopause are more prone to hypertension[9]. The higher prevalence of hypertension, and overall cardiovascular risk profiles, in postmenopausal women might be due to both aging and estrogen deficiency, with a strong multi-colinearity between the two events. Several reports have indicated that estrogens may alter levels of epinephrine and norepinephrine through different effects[10–15]. Consequently, HRT may modify the blood pressure increase related to aging, thus decreasing cardiovascular risk.

In addition, the significant positive effects exerted both on NOx and lipids suggest protective consequences on endothelial function. In fact, despite increased levels of triglycerides, a reduction of LDL cholesterol was observed. Previous studies have shown that HRT may favorably affect the profile of lipids in an anti-atherosclerotic direction[16,17]. These positive

Table 2 Changes in different physiological parameters observed in 15 healthy recently postmenopausal women before and during hormone replacement therapy (HRT). Data are presented as mean ± standard error

	Baseline	*1 month oral treatment*	*1 month transdermal treatment*	*6 months HRT*	*12 months HRT*	*p Value**
Systolic blood pressure (mmHg)	123 ± 3	122 ± 4	119 ± 3	118 ± 3	117 ± 3	0.078
Diastolic blood pressure (mmHg)	74 ± 2	77 ± 3	70 ± 3	67 ± 2	67 ± 2	0.009
Total cholesterol (mg/dl)	200 ± 7	193 ± 6	203 ± 6	193 ± 4	191 ± 9	0.3
HDL cholesterol (mg/dl)	62 ± 3	70 ± 4	64 ± 4	61 ± 3	64 ± 4	0.03
Triglycerides (mg/dl)	62 ± 6	78 ± 9	75 ± 9	85 ± 10	85 ± 9	0.013
LDL cholesterol (mg/dl)	126 ± 6	110 ± 5	122 ± 5	113 ± 4	117 ± 5	0.008
Glucose (mg/dl)	94 ± 2	92 ± 3	90 ± 3	93 ± 2	94 ± 3	0.7
Nitrite/nitrate (mmol/l)	75 ± 5	122 ± 13	94 ± 10	88 ± 7	93 ± 7	0.003
6-keto $PGF_{1\alpha}$ (pg/ml)	226 ± 26	275 ± 44	309 ± 38	346 ± 41	313 ± 38	0.058
8-isoprostane	243 ± 40	332 ± 69	307 ± 65	292 ± 37	310 ± 46	0.7

*Repeated measure ANOVA; HDL, high density lipoprotein; LDL, low density lipoprotein

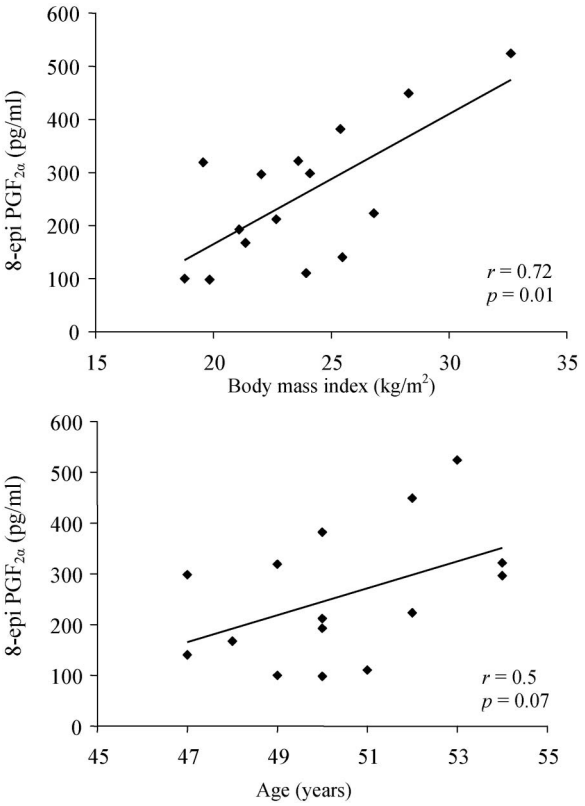

Figure 5 Regression analysis between 8-epi $PGF_{2\alpha}$ and body mass index and age in 15 healthy women in recent menopause

modifications induced by HRT in HDL and LDL cholesterol levels may have indirectly contributed to the increased vascular nitric oxide (NO) activity[7,18]. Otherwise, estradiol has been found to increase directly the activity of constitutive NO synthase in endothelial cells[19]. The increased NOx concentration that we observed, together with the positive trend of prostacyclin levels, may effectively contribute to the maintenance of a healthy endothelium.

A positive association between 8-isoprostane and body mass index and aging has been demonstrated[20,21]. Increased body mass index appears also to be associated with endothelial dysfunction and atherosclerosis[22]. We observed this association also in our study population of healthy women. Moreover, we have already shown a relationship between aging and 8-epi $PGF_{2\alpha}$ levels, in a population including patients with coronary artery disease[23]. Currently, although in a narrow age range (47–54 years), we also observed a positive trend between age and 8-isoprostane. By contrast, we did not detect any significant impact of HRT on 8-epi $PGF_{2\alpha}$ levels, suggesting that this treatment did not exert negative effects on the oxidative stress status. Accordingly, very recent data indicate that lipid peroxidation is not increased in postmenopausal women taking HRT compared to postmenopausal women without HRT[24].

In conclusion, we add evidence about the importance of the time of HRT initiation in terms of vascular benefit, which can really constitute a critical window of opportunity. In this context, this manuscript is well inserted in the animated discussion regarding controversial results raised after the publication of large randomized trials such as the Heart and Estrogen/progestin Replacement Study (HERS) and the Women's Health Initiative (WHI). In fact, these studies, reporting null or negative results, retain several biases. They

included older women, enrolled years after the onset of the menopause status, most of them without presenting menopausal symptoms, and showing signs of advanced atherosclerosis. The reality is quite different and these criteria do not reflect common clinical practice, because the majority of women start HRT for the relief of menopausal symptoms around 50 years. In addition, it is emerging that the timing of HRT is crucial at a vascular level, because women who are postmenopausal for more years are clearly exposed to a longer period of estrogen loss, which may lead to reduced number and activity of vascular estrogen receptors, contributing to the lack in efficacy of HRT. Moreover, they could have accumulated more extensive atherosclerotic injury or developed endothelium dysfunction, resulting in an overall reduced vascular responsiveness. At the moment, research into the effects of HRT on vascular biomarkers is lacking. We have demonstrated that HRT, including a cyclic progestin, initiated shortly after menopause in healthy women may exert a cardioprotective role and contribute to maintaining vascular health, improving vascular reactivity and possibly delaying clinical signs of atherosclerotic disease.

This knowledge is of particular importance because an increasing number of women are actually using or deciding to start this therapy, but opposing opinions and unclear information make this a difficult decision. Thus, better understanding will help physicians to individualize their prescribing of HRT, basing their decisions on the personal risk–benefit ratio rather than an extrapolation of epidemiologic results and supporting the woman in making an informed decision.

Conflict of interest Nil.

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