Hormone replacement therapy can augment vascular relaxation in post-menopausal women with type 2 diabetes

M.Perera1, J.R.Petrie2, C.Hillier3, M.Small4, N.Sattar5, J.M.C.Connell6 and M.A.Lumsden7,8

1Department of Obstetrics and Gynaecology, University of Glasgow, G3 8SJ, 2Department of Medicine and Therapeutics, Western Infirmary, University of Glasgow, G11 6NT, 3Vascular assessment unit, Glasgow Caledonian University, G12 8QQ, 4Diabetes Centre, Gartnavel General Hospital, North Glasgow Hospitals University NHS Trust, 5Department of Pathological Biochemistry, University of Glasgow, G31 2ER, 6Department of Medicine and Therapeutics, Western Infirmary, University of Glasgow, G11 6NT and 7Department of Obstetrics and Gynaecology, University of Glasgow, Glasgow, UK

E-mail: M.A.Lumsden@clinmed.gla.ac.uk

BACKGROUND: Diabetes is a major risk factor for coronary heart disease in women and event rates increase substantially after the menopause. Observational studies have suggested that estrogens may provide cardioprotection by regulating endothelial nitric oxide synthase. METHODS: In order to examine the effect of hormone replacement therapy (HRT) on endothelium-dependent and -independent vascular relaxation in post-menopausal women with type 2 diabetes, an open study was conducted in which gluteal biopsies were collected from 17 women before and after 6 months of transdermal 17β-estradiol (80 µg twice weekly) in combination with oral norethisterone (1 mg daily). Small arteries (<550 µm) were dissected from fat and mounted on a wire myograph for assessment of relaxation in response to acetylcholine (ACh), bradykinin (BK) and sodium nitroprusside (SNP). RESULTS: Maximal relaxation responses to ACh, BK and SNP in women with diabetes and non-diabetic control subjects were 52 ± 8 versus 96 ± 2% (P < 0.05), 76 ± 7 versus 97 ± 1%, (P < 0.05) and 91 ± 2 versus 98 ± 1% (P < 0.05) respectively. After 6 months of HRT, maximal relaxation responses to ACh, BK and SNP in women with diabetes (compared with pre-HRT) were: 88 ± 4 (P < 0.05), 93 ± 3 (P < 0.05) and 98 ± 1% (P < 0.05) respectively. At baseline and after HRT, EC50 (concentration required to obtain 50% of maximum response) data exhibited similar changes. CONCLUSIONS: HRT had potentially beneficial effects on vascular relaxation. Data were consistent with improvements in endothelial function, vascular smooth muscle function, or both. Controlled studies are required to confirm and extend these findings.

Key words: endothelium/hormone replacement therapy/nitric oxide/type 2 diabetes

Introduction

Coronary heart disease (CHD) is the most important cause of death in type 2 diabetes mellitus. Middle-aged women with diabetes have a five times greater risk of CHD than their male counterparts, with a relative risk of death from CHD of 1.5–2.5 in men (Kessler, 1971; Kannel and McGee, 1979) and 1.7–4.0 in women (Barrett-Connor et al., 1991).

A number of inter-related phenotypic abnormalities underpinned by resistance to insulin-mediated glucose uptake including hyperglycaemia, hypertension, dyslipidaemia, and a prothrombotic state (Cleland et al., 1998) are linked via oxidative stress (Stehouwer et al., 1997; Laight et al., 1999) and inflammation (Pickup et al., 1997) with accelerated CHD in type 2 diabetes. Endothelial dysfunction plays a central role in these processes (Nitenberg et al., 1993; Williams et al., 1996). A growing body of evidence suggests that the vascular endothelium in type 2 diabetes produces abnormally raised levels of vasoconstrictors (Nugent et al., 1996; Laight et al., 1999) and procoagulants (Collier et al., 1992) and has a reduced capacity to synthesize nitric oxide (NO) and other vasodilators (Hsueh et al., 1997; Honing et al., 1998). In experimental models of atherosclerosis, reduced formation of NO is compounded by increased endothelial production of superoxide by enzymes including NO synthase, particularly under conditions of co-factor deficiency (Beckman and Koppenol, 1996; Wever et al., 1997). This, in turn, reacts with NO to form peroxynitrite, which may disrupt metabolic processes in adjacent tissues (Pryor and Squadrito, 1995). These metabolic perturbations may increase peripheral resistance, decrease tissue perfusion and ultimately contribute to the development of CHD and other forms of cardiovascular disease.

In non-diabetic women prior to the menopause it has been suggested on the basis of observational data that hormone replacement therapy (HRT) may be cardioprotective (Grodstein et al., 1996). Basal release of NO from coronary endothelial cells, known to control basal vascular tone and antagonize
prothrombotic and proinflammatory processes (Vallance, 1998), is enhanced by estrogen which can also up-regulate endothelial NO synthase (Caulin-Glaser et al., 1997). The only large randomized placebo-controlled study to date examining the effects of estrogen in the prevention of CHD is the Heart and Estrogen/progestin Replacement Study (HERS) which was conducted in relatively elderly women (mean age 67 years) in the secondary prevention setting (Hulley et al., 1998). No overall beneficial effect of the HRT regimen based on conjugated equine estrogens and medroxyprogesterone acetate was detected and an early excess of thrombotic events was observed. There are as yet no randomized data on primary prevention of CHD in middle-aged women.

In addition to increasing NO synthesis, estrogen has potentially favourable effects on lipoprotein profiles (Andersson et al., 1997), carbohydrate metabolism (Brussard et al., 1997) and vascular reactivity (Gilligan et al., 1994; Mendelsohn and Karas, 1999). As multiple pathophysiological processes contribute to coronary atherosclerosis and thrombosis in type 2 diabetes, it is important that the effects of different forms of HRT on each of these processes are delineated in order to optimize design of future HRT-based primary prevention studies in type 2 diabetes.

We have recently suggested that HRT regimens based on 17β-estradiol and progesterone (norethisterone) may be more rational for women with diabetes than those based on synthetic conjugated estrogens (Sattar et al., 1999a,b). In addition, we have hypothesized that women with type 2 diabetes stand to beneﬁt more from any HRT cardioprotection than their non-diabetic counterparts on the basis of their higher absolute baseline risk (Sattar et al., 1998). However, owing mainly to concerns regarding effects on glycaemic control, diabetes remains listed as a relative contraindication to HRT and rates of prescription and uptake are low (Feher and Isaacs, 1996).

In the present study, wire myography was used to assess the effect on small vessel endothelial function of 6 months of continuous combined HRT, consisting of transdermal 17β-estradiol and oral norethisterone, in women with type 2 diabetes.

Methods

The study conformed to the principles outlined in the Declaration of Helsinki and was approved by the local ethical review committee of the West Glasgow Hospitals University NHS Trust.

Patients and control subjects

All subjects gave prior written, informed consent. Seventeen postmenopausal women with type 2 diabetes, good glycaemic control [haemoglobin (Hb)A1c <6.5%], blood pressure (BP) <150/95 mmHg and total cholesterol <5.2 mmol/l were recruited from clinics at the Diabetes Centre, Gartnavel General Hospital, Glasgow. Three non-diabetic women of similar age, body mass index (BMI) and BP were also studied. None of the subjects had previously received HRT and all had been amenorrheic for at least one year. None had evidence of microvascular complications, microalbuminuria or symptoms of ischaemic heart disease. None were on statin therapy. Those on aspirin and/or stable monotherapy with metformin, sulphonylureas or dihydropyridines only were included and remained on these treatments throughout the trial with no change in dose.

Each woman was invited to attend for gluteal biopsy before and after 6 months of treatment with continuous combined HRT consisting of 80 μg estradiol patches applied twice weekly in combination with 1 mg oral norethisterone daily. Pharmacological assessment of vascular function was assessed ex vivo using wire myography on the same day as biopsy after dissection of subcutaneous resistance arteries.

Subcutaneous gluteal fat biopsies

Skin and subcutaneous fat biopsies were taken as previously described (McNally et al., 1995; Padmanabhan et al., 1999) after application of 1% lidocaine local anaesthesia, from the right (pre-HRT) and left (post-HRT) gluteal region. Each biopsy measured approximately 2×1×1 cm.

Vessel preparation

Abnormal endothelial function has its greatest effect on the circulation at the level of the resistance vasculature (100–400μm) at which point a 50% drop in pre-capillary pressure occurs (Davis et al., 1986). Small arteries (<550μm) were therefore dissected from biopsied fat under a microscope and mounted on two 40 μm stainless steel wires on a Mulvany/Halpern myograph allowing isometric force measurements to be made (Davis et al., 1986). Where possible, two or three vessels from each patient were investigated simultaneously under identical conditions. The vessels were maintained at 37 ± 0.5°C in 95% O2/5% CO2 to a pH of 7.4 in physiological salt solution (PSS) with the following composition: NaCl 118.4, NaHCO3 25, KCl 5.2, KH2PO4 1.2, CaCl2 2.5, MgSO47H2O 1.2 and glucose 11 mmol/l.

Wire myography

The method used was that of Mulvany (Mulvany et al., 1978). Following an equilibration period, each vessel was stretched in a step-wise fashion to allow an estimate of the transmural pressure required to stretch the relaxed vessel in vivo to the given internal circumference. Finally, each vessel was set to 90% of its own internal circumference at an effective pressure of 100 mmHg (previous studies have shown that a maximal force is generated at this setting) (Mulvany et al., 1978). Following this normalization procedure, the vessels were left for a further 1 h and then exposed to a high (123 mmol/l) concentration of potassium (solution identical to PSS except that sodium is replaced by potassium on an equimolar basis) for a series of 5 min periods until repeatable maximal contractions were achieved.

Acetylcholine (ACh) and Bradykinin (BK) act via receptor-mediated mechanisms within endothelial cells to promote synthesis/release of NO which activates guanylate cyclase on adjacent smooth muscle to cause vascular relaxation. Sodium nitroprusside (SNP) donates NO directly to smooth muscle cells and therefore does not require the presence of intact endothelial mechanisms. Cumulative concentration response curves were carried out according to the following protocol:

(i) noradrenaline (NA): 10^-8 to 3×10^-5 mol/l
(ii) ACh (10^-8 to 10^-4 mol/l) then BK (10^-10 to 3×10^-6 mol/l) in preconstricted vessels (NA: 10^-5 mol/l)
(iii) SNP (10^-9 to 10^-4 mol/l) in preconstricted vessels (NA: 10^-5 mol/l).

Vessels were left for 30 min between assessment of dose–response curves. It has previously been shown that contraction responses of human vessels to noradrenaline are stable over 6 h and that 30 min between curves is sufficient to achieve repeatable contraction responses (Hillier et al., 1999).

Drugs

ACh, BK, NA and SNP were obtained from the Sigma Chemical Company, Poole, Dorset, UK. All drugs were dissolved in distilled water and diluted to the final bath concentration with PSS.
HRT and endothelial function in type 2 diabetes

Figure 1. Vascular relaxation (expressed as percentage change from maximum noradrenaline-induced contraction) to acetylcholine (ACh) in resistance arteries from control subjects and from women with type 2 diabetes before and after continuous combined HRT. Significant difference between pre-HRT and control, $P < 0.01$.

**Statistical analysis**
Relaxation data are expressed as percentage change (%) from maximum noradrenaline-induced contraction. Where more than one vessel from a single biopsy was successfully mounted, data were treated as replicates and a mean response used in further analysis. Results are expressed as mean ± SEM. Maximal relaxation response and EC$_{50}$ (concentration required to obtain 50% of maximum response) were used as summary measures (Matthews et al., 1990). Statistical comparison was performed after checking appropriate distribution of data using unpaired (control versus pre- or post-HRT) and paired (pre- versus post-HRT) $t$-tests with $P < 0.05$ considered statistically significant. Simple correlation was used to examine relationships between vascular responses and baseline demographic and metabolic data.

**Results**
Characteristics of women recruited to the study were as follows: age 61 ± 3.7 years, BMI 30.3 ± 7.03 kg/m$^2$, HbA1c 6.2 ± 1.25%, total cholesterol 5.5 ± 0.94 mmol/l, blood pressure (BP) systolic/diastolic 130 ± 18.1/86 ± 13.1 mmHg). Prior to treatment, 26 vessels from 17 biopsies (i.e. nine replicates) from women with type 2 diabetes were dissected and successfully mounted, while six vessels were obtained for comparison from the three non-diabetic women. Biopsies were well-tolerated and all women consented to a repeat procedure. At follow-up, 36 vessels were obtained from 17 biopsies from the same women. BMI and glycaemic control were similar at baseline and follow-up.

**Pre-HRT**
In comparison with vessels from control subjects, vessels from women with type 2 diabetes exhibited impaired vasorelaxation to both endothelium-dependent and endothelium-independent agonists (Table I, Figures 1–3). No correlation was observed between vasorelaxation responses and HbA1c or total cholesterol. There was a trend towards improved relaxation in women with a BMI >30 m/kg$^2$. However, the small numbers prevent further meaningful analysis of the results at this stage.

**Post-HRT**
Following 6 months HRT, responses in arteries from patients with type 2 diabetes were augmented compared with baseline responses (Table I, Figures 1–3). Post-HRT responses were similar to those observed in vessels from control subjects.

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<th>Table I. Maximal relaxation responses and EC$_{50}$ for control subjects and women with diabetes pre- and post-HRT</th>
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<td>Acetylcholine</td>
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$^aP < 0.05$ compared with control.

$^bP < 0.05$ compared with pre-HRT.

Figure 2. Vascular relaxation (expressed as percentage change from maximum noradrenaline-induced contraction) to bradykinin (BK) in resistance arteries from control subjects and from women with type 2 diabetes before and after continuous combined HRT. Significant difference between pre-HRT and control, $P < 0.01$.

Figure 3. Vascular relaxation (expressed as percentage change from maximum noradrenaline-induced contraction) to sodium nitroprusside (SNP) in resistance arteries from control subjects and from women with type 2 diabetes before and after continuous combined HRT. Significant difference between pre-HRT and control, $P < 0.01$. 

$^aP < 0.05$ compared with control.

$^bP < 0.05$ compared with pre-HRT.
Discussion

In the present study we observed augmentation of responses *ex vivo* to both endothelium-dependent and -independent agonists in subcutaneous resistance arteries from post-menopausal women with type 2 diabetes treated with continuous combined HRT for 6 months. Indeed, responses after HRT to all agonists were similar to those observed in a small number of non-diabetic control subjects. The limitations of these data are recognized, but recruiting non-diabetic women to act as control subjects in this type of study is extremely difficult.

Post-menopausal women with type 2 diabetes are at high risk of CHD and the importance of endothelial dysfunction in atherogenesis and thrombosis is increasingly recognized (Ross, 1993). The finding of impaired responses to both endothelium-dependent and -independent agonists was not unexpected. It is now acknowledged on the basis of studies using a variety of invasive and non-invasive techniques (forearm plethysmography (McVeigh *et al.*, 1992; Williams *et al.*, 1996), flow-mediated vasodilation (Goodfellow *et al.*, 1996), laser-doppler iontophoresis (Morris *et al.*, 1995) and wire myography (Cipolla *et al.*, 1996) that those with type 2 diabetes have dysfunctional endothelium in both conduit and resistance arteries. Indeed, this also appears to be the case in insulin-resistant first degree relatives (Caballero *et al.*, 1999; Balletshofer *et al.*, 2000).

Abnormal responses to endothelium-independent agonists have been reported in type 2 diabetes by other workers (Flavahan, 1992; McVeigh *et al.*, 1992; Williams *et al.*, 1996). In the current study it was found that vessels from women with diabetes were 20-fold less sensitive to SNP than those of disease duration with progressive vascular damage affecting SNP (Flavahan, 1992). However, in the present study, SNP had been reported in type 2 diabetes by other workers (Cipolla *et al.*, 1996; Balletshofer *et al.*, 1999). Interpretation of abnormal ‘endothelium-dependent’ responses is difficult in this situation, as the defect cannot be attributed to the endothelial monolayer. It has been suggested that heterogeneity of responses to sodium nitroprusside between studies can be explained on the basis of disease duration with progressive vascular damage affecting ACh responses and subsequently those to BK and finally SNP (Flavahan, 1992). However, in the present study, SNP responses were impaired to a much greater extent than those to BK.

In contradistinction to the notion of progressive vascular damage, the subjects had low HbA1c values and a median disease duration of only 3 years. However, type 2 diabetes is often preceded by years of insulin resistance and glucose intolerance (Goyder and Irwig, 1998). It may be the case that endothelium-derived mediators such as peroxynitrite, formed under conditions of oxidative stress, result in damage to the smooth muscle layer at a relatively early stage in the course of diagnosed disease. Furthermore, it is now recognized that advanced glycosylation end-products are deposited in a wide variety of tissues over a relatively short time course (Schmidt *et al.*, 1999). This represents an alternative possible mechanism for abnormal endothelium-independent responses, although the reversibility of abnormal responses observed over 6 months in the present study favours a functional rather than structural explanation. A further possible mechanism is an abnormality at the level of smooth muscle guanylate cyclase, as has recently been proposed by Reaven (Zavaroni *et al.*, 2000).

Circumstantial evidence is in favour of a positive effect of sex hormones on vascular function. For example, the rate of decline in acetylcholine vasodilatation with age is attenuated in premenopausal women in a manner that appears to mirror protection from CHD (Taddei *et al.*, 1996). Intra-brachial responses to the NO synthase inhibitor NG-monomethyl-L-arginine (L-NMMA) were augmented in non-diabetic post-menopausal women after a brief period of HRT (Sudhir *et al.*, 1996). However, it appears that there have been no previous reports of augmented endothelium-independent responses after HRT in women with or without type 2 diabetes.

It could be possible that the augmentation of vascular responses observed after HRT in the present study was not a genuine effect. Owing to the nature of the design, bias cannot formally be excluded—although it should be noted that true double-blinding is notoriously difficult in any study of HRT versus placebo. As formal time controls were not incorporated, it remains conceivable that the effect observed was an artefact of a confounding and unrecognized technical factor. However, variations in responses to acetylcholine, sodium nitroprusside and bradykinin in previous studies have been small when compared with the effect size reported herein (Hillier *et al.*, 1999). Another technical factor worthy of consideration, which may influence results, was the preconstriction agent. The major difference between that study and the present one was use of thromboxane rather than noradrenaline for preconstriction; it is uncertain which approach is more physiologically relevant.

Women participating in the present study continued on aspirin and stable monotherapy with sulphonylureas, metformin or dihydropyridines. Even if it had been considered ethical to discontinue such medication over a 6 month period, the data would have been both subject to carryover effects and would not be applicable to the majority of women with type 2 diabetes. In women who continued on aspirin, inhibition of the cyclo-oxygenase pathway may have made vascular relaxation responses to acetylcholine smaller and more NO-dependent than in other subjects, but this would not have affected the pre- and post-treatment comparison. Although there was a tendency for the vascular reactivity to be improved in the obese women (BMI >30m/kg²), there was no weight change in the women during the study, and so this factor was not thought to influence the results. It will be of interest to investigate this further in the future.

If HRT did augment relaxation to endothelium-dependent agonists, possible mechanisms for this include up-regulation of endothelial NO synthase (Sudhir *et al.*, 1996; Caulin-Glaser *et al.*, 1997), an indirect effect on endothelial function by virtue of anti-inflammatory properties or favourable effects on insulin sensitivity, lipoprotein profiles and oxidative stress or a reduction in endothelin-1. In addition, a direct or indirect effect leading to increased NO bioavailability by inhibiting its metabolism (Wever *et al.*, 1997) is under investigation. These hypotheses cannot be addressed within the design of the present study.

Data from a study by our own group, taken together with data from the literature (Sattar *et al.*, 1999a,b) indicate that
norethisterone has heptatically-mediated anti-inflammatory effects that may, in turn, have downstream favourable effects on endothelial function. In keeping with this notion, it has recently been reported that C-reactive protein levels—an index of chronic, low grade inflammation—show high correlation with basal endothelial NO synthesis/release (Cleland et al., 2000). Inflammation is increasingly recognized as a determinant of CHD risk (Ridker et al., 1997) and it is attractive to speculate that anti-inflammatory properties of different HRT preparations may be relevant to their effects on CHD risk.

The observation of reduced sensitivity to bradykinin in type 2 diabetes may be clinically relevant to the beneficial effects of angiotsin converting enzyme (ACE) inhibitors in type 2 diabetes (Anonymous, 2000). Unlike ACh, BK is an endogenous vasodilator with a functional role in modulating vascular tone in resistance arteries. There is some evidence that estrogen can upregulate the endothelial renin-angiotensin system and thereby potentiate BK responses. Recent descriptions of non-ACE pathways for angiotensin II formation have recently re-emphasised the potential cardiovascular importance of BK potentiation rather than inhibition of angiotensin II formation during ACE inhibition (Mulvany et al., 1978). Thus, reducing BK breakdown in patients with type 2 diabetes via ACE inhibition might provide a complementary strategy to HRT for restoring endothelial function.

Recent studies suggest that a major component of relaxation responses to both BK and ACh is dependent on release of a derived hyperpolarizing factor (EDHF) (Coats et al., 2000), whose role may be in influenced by female sex steroids (Hansen et al., 1997) and it is attractive to speculate of CHD risk (Ridker et al., 1997) and it is attractive to speculate that anti-inflammatory properties of different HRT preparations may be relevant to their effects on CHD risk. of CHD risk—of CHD risk (Ridker et al., 1997) and it is attractive to speculate that anti-inflammatory properties of different HRT preparations may be relevant to their effects on CHD risk. of CHD risk—of CHD risk (Ridker et al., 1997) and it is attractive to speculate that anti-inflammatory properties of different HRT preparations may be relevant to their effects on CHD risk. 

In summary, in this small study utilizing careful ex vivo assessments, HRT consisting of continuous combined transdermal 17β-estradiol and oral norethisterone resulted in potentially beneficial effects on vascular relaxation in postmenopausal women with type 2 diabetes. Data were consistent with improvements in endothelial function, vascular smooth muscle function, or both. On the basis of the present data, HRT regimen consisting of transdermal estradiol with continuous oral norethisterone are attractive candidates to enter primary prevention CHD trials in post-menopausal women with type 2 diabetes.

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