Aortic function is compromised in a rat model of polycystic ovary syndrome

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BACKGROUND: Arterial mechanical parameters are modified in women with polycystic ovary syndrome (PCOS), before and during pregnancy. This study tested the hypothesis that aortic mechanics and endothelial function are modified in the mifepristone-treated rat model of PCOS. METHODS: Female rats injected daily with mifepristone or vehicle for 7–9 days were assessed by ultrasound to allow estimation of aortic stiffness index and compliance. The influence of acetylcholine (ACh) and sodium nitroprusside (SNP) on dissected phenylephrine-contracted aortic rings was assessed. RESULTS: Aortic compliance was reduced by 67% in mifepristone-treated rats versus controls ($P < 0.05$), while stiffness index was increased 2.3-fold ($P < 0.02$). ACh-induced dilation was less in aortic rings from mifepristone-treated rats ($P = 0.022$) and was less sensitive to the nitric oxide (NO) synthase inhibitor N6-nitro-L-arginine methyl ester (L-NAME) ($P < 0.001$), while SNP-induced dilation was greater ($P = 0.001$). CONCLUSIONS: Aortic mechanics in vivo and endothelial function in vitro were consistently perturbed in mifepristone-treated rats. Aortic ring behaviour suggested that NO release was depressed or degradation elevated, with a compensatory increase in NO sensitivity and/or activation of a non-NO-mediated relaxation mechanism. The mifepristone-treated rat is a valid model for investigation of the vascular deficits seen in PCOS.

Key words: animal model/polycystic ovaries/ultrasound/vascular system

Introduction
Polycystic ovary syndrome (PCOS), characterized by infertility, oligo/amenorrhoea and hyperandrogenism, affects 5–10% of women of reproductive age. PCOS is also associated with metabolic abnormalities central to metabolic syndrome, such as hyperinsulinaemia, insulin resistance, impaired glucose tolerance and dyslipidaemia (Reaven, 2002; Isomaa, 2003), and with type II non-insulin-dependent diabetes mellitus (NIDDM) (Talbott et al., 1995). NIDDM is an independent risk factor for atherosclerosis (Kannel and McGee, 1979) and many of the metabolic abnormalities are predictors for cardiovascular disease (Conway et al., 1992; Reaven, 2002). An increased prevalence of cardiovascular disease is expected in women with PCOS and we have demonstrated increased vascular stiffness (Lakhani et al., 2002) and intima–media thickness in the carotid and femoral artery (Lakhani et al., 2003) in young women with PCOS, suggesting atherosclerosis and underlying endothelial dysfunction (Balletshofer et al., 2003; Plutzky, 2003). Indeed, evidence of endothelial dysfunction in PCOS has been observed in vivo in the femoral artery (Paradisi et al., 2003) and the microvasculature (Kelly et al., 2003; Lakhani et al., 2005), though not in the brachial artery (Mather et al., 2000). Recently we noted increased stiffness and decreased compliance in the carotid arteries of pregnant women with PCOS in the first trimester and beyond (Hu et al., 2004). Decreased arterial compliance in the first trimester is associated with a history of pre-eclampsia, the most common serious complication of pregnancy (Spaanderman et al., 2000; Redman and Sargent, 2005), while a history of pre-eclampsia, obesity and hyperinsulinaemia are associated with further pre-eclampsic episodes (Duckitt and Harrington, 2005). Thus women with PCOS are at increased risk of hypertensive disease during pregnancy, and their babies of morbidity and mortality related to pre-term delivery and intrauterine growth restriction (de Vries et al., 1998).

The mechanisms by which PCOS modifies vascular function are poorly understood and require urgent investigation. An animal model of PCOS would allow in vivo analysis of arterial function and in vitro investigation of endothelial function. In the rat, daily injections of the antiprogestin mifepristone (also
known as RU486) for ≥1 week induce features characteristic of PCOS in humans, for example ovulatory failure, persistent vaginal cornification, and enlarged ovaries containing atretic follicles and follicular cysts (Sánchez-Criado et al., 1993; Ruiz et al., 1996) as well as increased serum LH, testosterone and estradiol, and decreased prolactin (Sánchez-Criado et al., 1992, 1993; Ruiz et al., 1996, 1997). Serum insulin-like growth factor I levels are also increased (Ruiz et al., 1997) and the response of such rats to therapies used in the treatment of PCOS is similar to that in humans (Ruiz et al., 1996). The mifepristone treated rat is a ‘fundamentally adequate’ model of PCOS (Ruiz et al., 1996) with which to investigate the effect of PCOS-like endocrinological perturbations on other physiological parameters in the short term (1–2 weeks).

We have used this model to test the hypothesis that arterial mechanics and endothelial function are modified in PCOS due to the underlying endocrine dysfunction, since it allowed in vivo assessment of the mechanical properties of the aorta followed by precise in vitro assessment of endothelial and vascular smooth muscle function.

Materials and methods

All chemicals were obtained from Sigma–Aldrich Chemicals, Gillingham, UK, unless otherwise stated. Animal procedures were performed in compliance with the Animals (Scientific Procedures) Act 1986. Adult female Sprague–Dawley rats (aged 12–14 weeks, body weight 242 ± 4.5 g) were bred and housed locally at 22°C under a 14 h on/10 h off light cycle, with free access to food and water. Rats showing at least two consecutive 4–5 day estrous cycles were used, as assessed on a 1/10 h light cycle, with free access to food and water. Rats showing compliance with the Animals (Scientific Procedures) Act 1986, UK, unless otherwise stated. Animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, UK, unless otherwise stated. Animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, UK, unless otherwise stated. Animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, UK, unless otherwise stated. Animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, UK, unless otherwise stated.

Aortic function was assessed in vitro by measuring acetylcholine (ACH)-induced dilation in phenylephrine (PE)-contracted aortic rings, since this process requires functional interaction between the endothelium and smooth muscle. Aortae were washed several times in fresh Krebs buffer and cut into 2 mm wide rings, which were mounted on two hooks in a 7 ml organ bath containing Krebs buffer at 37°C, gassed with 95% O2/5% CO2. Isometric tension was measured with a transducer (Grass Instruments, Quincy, MA, USA) and digitized using a multi-channel recording system (Linton Instrumentation, Diss, Norfolk, UK) with AcqKnowledge ACK100W software (Biopac Systems Inc., Goleta, CA, USA), which allowed simultaneous testing of six rings per aorta. Rings were tensioned to ~1 g, equilibrated for 60 min, and contracted with 3 μmol/l PE. The dilatory response to cumulative doses of ACh (1 μmol/l–100 μmol/l) was then measured. The PE–ACh treatment was repeated, then again after 100 μmol/l Nω-nitro-L-arginine methyl ester (lNAME) was added to three of the six aortic rings. A final treatment cycle used the nitric oxide (NO) donor sodium nitroprusside (SNP; 1 μmol/l–100 μmol/l) instead of ACh, lNAME being added to the same three rings (Li and Forstermann 2000). Aortic tension was expressed as percentage relaxation, such that the tension induced by 3 μmol/l PE was defined as 0% relaxation, and the tension prior to PE treatment was defined as 100% relaxation.

Serum LH, FSH and insulin were assayed in duplicate by enzyme-linked immunonassay using Biotrak kits (APBiotech, Amersham, UK), with coefficients of variation (CV) of 7.6, 11.4 and 13.8% respectively. Serum testosterone was determined using an enzyme-linked immunosorbent assay kit (Roche Diagnostics, Welwyn Garden City, Herts, UK) with an intra-assay CV of 11.7%. Ovarian morphology was assessed in 6 μm sections cut from the wax-embedded ovary, to determine the presence of features consistent with previous studies of mifepristone-treated rats (Sánchez-Criado et al., 1993; Ruiz et al., 1996).

The aortic diametrical compliance (C) and stiffness index (β) were calculated using the mean aortic wall movement over at least three cardiac cycles and the aortic blood pressure and flow estimates taken immediately after laparotomy (Lakhani et al., 2002). Statistical significance (P < 0.05) was tested by analysis of variance (ANOVA) with post hoc analysis by Fisher’s protected least significant difference (PLSD) test. All data are expressed as mean ± SEM.

Results

Treatment of female rats with mifepristone for 7–9 days had no significant effect on body weight, SBP, DBP or mean arterial pressure (Table I). Endocrine disturbances were apparent (Table I), as previously noted (Sánchez-Criado et al., 1993; Ruiz et al., 1996). Mifepristone-treated rats exhibited increased serum LH and testosterone. Serum insulin also tended to be elevated, but not significantly due to hypervariable insulin concentrations in the treated rats (Table I). Mifepristone treatment had no effect on serum FSH. Mifepristone-treated ovaries exhibited evidence of an increase in the abundance of follicular cysts and of arrested follicular growth (Figure 1), as previously reported (Sánchez-Criado et al., 1993; Ruiz et al., 1996).

CV for ultrasound estimates of aortic parameters were in nearly all cases <10%, indicating that measurements were...
made reproducibly. In mifepristone-treated animals, the mean aortic diameter and blood flow were unaffected; however, aortic compliance was reduced by 67% while the stiffness index was increased 2.3-fold, relative to controls (Table II).

In vitro organ bath assessment of endothelial and smooth muscle function was performed to determine whether the changes in aortic compliance and stiffness were related to dysfunction in these tissues. The PE-induced contraction (post 3 μmol/l PE tension—resting tension) was decreased in the mifepristone-treated animals compared to the controls (349 ± 34 and 781 ± 126 mg respectively, \(P < 0.001\)). ACh induced a concentration-dependent relaxation in PE-contracted aortic rings from mifepristone-treated and control animals (\(P < 0.001\), ACh effect by two-way ANOVA; Figure 2); however, relaxation was less in mifepristone-treated animals compared to controls (\(P = 0.022\), mifepristone treatment effect by two-way ANOVA), notably at 0.1 μmol/l and 1.0 μmol/l ACh (Figure 2). This difference was not reflected by changes in the maximal relaxation (76 versus 81% at 100 μmol/l ACh, mifepristone-treated versus control) or in \(E_{D50}\) (i.e. –log10 of the concentration producing a half-maximal response), which was 7.9 (95% CI, 7.8–8.0) in mifepristone-treated rat aortic rings and 7.8 (95% CI 7.7–8.1) in controls.

ACh-stimulated aortic relaxation is endothelium dependent, and thought to be due to activation of endothelial nitric oxide synthase (eNOS), producing nitric oxide (NO), which diffuses to the underlying smooth muscle causing relaxation. Indeed L-NAME, which inhibits 80% of eNOS activity, considerably impaired ACh-induced relaxation in aortic rings from normal and mifepristone-treated animals (\(P < 0.001\), L-NAME treatment effect in both the mifepristone-treated and control groups by 2-way ANOVA; Figure 2). The inhibitory effect of L-NAME on ACh-induced relaxation was, however, less in aortic rings from the mifepristone-treated rats than in controls (\(P < 0.001\), effect of mifepristone treatment on ACh induced/ L-NAME antagonized relaxation by two-way ANOVA), notably at ACh concentrations of ≥1 μmol/l (Figure 2). This difference was reflected by a change in the maximal relaxation produced by 100 μmol/l ACh after 100 μmol/l L-NAME blockade (35% versus 16%, \(P < 0.005\), mifepristone-treated versus control), but not in \(E_{D50}\) (7.1 [95% confidence interval (CI), 6.5–7.5] versus 7.1 (95% CI 6.5–7.6), mifepristone-treated versus control).

To determine smooth muscle function independent of endothelial NO, aortic dilation due to smooth muscle relaxation was assessed using cumulative doses of SNP, a non-endothelium-dependent NO donor. As for ACh, SNP induced a concentration-dependent \(P < 0.001\), SNP effect by two-way ANOVA) relaxation in aortic rings from mifepristone-treated and control animals (Figure 3). Relaxation was, however, greater in mifepristone-treated animals compared to controls.

### Table I. Physical and endocrine parameters in mifepristone-treated rats and control rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Mifepristone</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>238.3 ± 8.3</td>
<td>244.8 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>62.0 ± 1.6</td>
<td>74.4 ± 6.6</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>54.3 ± 3.3</td>
<td>59.8 ± 5.8</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>56.9 ± 2.7</td>
<td>64.6 ± 5.8</td>
<td>NS</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>9.35</td>
<td>15.1 ± 1.46</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>0.47 ± 0.10</td>
<td>1.81 ± 0.51</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Insulin (mIU/l)</td>
<td>0.76 ± 0.22</td>
<td>1.44 ± 0.41</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (ng/l)</td>
<td>150.0 ± 30.1</td>
<td>143.5 ± 20.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; NS = not significant.

### Table II. Aortic diameter, blood flow and mechanical parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mifepristone</th>
<th>Control</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (mm)</td>
<td>1.44 ± 0.04</td>
<td>1.32 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Blood flow (ml/min)</td>
<td>32.0 ± 2.6</td>
<td>37.0 ± 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Compliance (%mm Hg⁻¹×10⁻²)</td>
<td>140.9 ± 6.3</td>
<td>426.9 ± 17.4</td>
<td>0.05</td>
</tr>
<tr>
<td>Stiffness index</td>
<td>1.30 ± 0.07</td>
<td>0.57 ± 0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NS = not significant.
This was reflected by a change in the ED 50 for SNP from 7.4 μmol/l (95% CI 7.1–7.6) in mifepristone-treated rat aortic rings to 8.1 (95% CI 7.9–8.2) in controls, but not in the maximal relaxation produced by 0.1 mmol/l SNP (103 versus 105%, mifepristone-treated versus control).

**Discussion**

These results demonstrate increased stiffness index and decreased compliance in the aorta in vivo in this rat model of PCOS, replicating the changes seen in PCOS women, albeit in other arteries (Lakhani et al., 2002, 2003; Kelly et al., 2003). The altered arterial mechanics have important implications in women with PCOS if they conceive, as the reduced arterial compliance persists in the first trimester and beyond (Hu et al., 2004) and is associated with a history and further risk of pre-eclampsia (Spaanderman et al., 2000; Duckitt and Harrington, 2005). These findings are compatible with reports of an association between PCOS and hypertensive disorders of pregnancy (de Vries et al., 1998), a hypothesis we are currently investigating.

ACh-stimulated endothelium-dependent relaxation was attenuated in aortic rings from mifepristone-treated rats, whereas endothelium-independent SNP relaxation was exaggerated. L-NAME partially inhibited ACh relaxation, indicating it to be NO-mediated. Residual relaxation may be due to non-NO-mediated mechanisms or because L-NAME inhibits only 80% of eNOS activity (Li and Forstermann, 2000). L-NAME inhibition was reduced in aortic rings from mifepristone-treated rats, perhaps because a non-NO-mediated relaxation mechanism was more active. The mifepristone-related changes in aortic stiffness and compliance in vivo were consistent with the effects on aortic dilation noted in vitro. The endothelium synthesizes many vasodilators, NO being prominent in the rat aorta, with prostacyclin having a lesser role and endothelium-derived hyperpolarizing factor (EDHF) being more active in smaller arteries (Palmer et al., 1987; Shimokowa et al., 1996; Ge and He, 2000). This study suggests that aortic NO synthesis is impaired, and/or NO degradation enhanced, in mifepristone-treated animals. In partial compensation, aortic smooth muscle is more sensitive to NO, and non-NO-mediated ACh-stimulated vasodilation is more active, perhaps based on EDHF or prostacyclin.

Changes in endothelial behaviour and aortic mechanics may result from the endocrine effects of mifepristone injection. Serum estradiol is elevated in this model (Sánchez-Criado et al., 1993; Ruiz et al., 1996) but this should enhance, not diminish, vasodilation since estradiol stimulates endothelial NOS expression and NO synthesis (Goetz et al., 1994; Andersen et al., 1999), and ACh-induced aortic relaxation (Huang et al., 1998; Teoh et al., 2000). The elevated serum testosterone in mifepristone-treated rats could diminish vasodilatory responses to ACh, since in PCOS women, leg blood flow response to methacholine is negatively correlated with serum testosterone (Steinberg et al., 1996; Paradisi et al., 2003). The causal link is perhaps with insulin resistance, as noted in women with NIDDM, due to its effect on arterial smooth muscle NO sensitivity and degradation (Williams et al., 1996). In the present study, mifepristone had no effect on serum insulin and it enhanced aortic ring SNP sensitivity; nevertheless NO degradation might be increased. Testosterone stimulates aortic ring prostanoid synthesis and dilation in vitro (Selles et al., 2002; Tep-areenan et al., 2003). This cannot explain the diminished ACh dilation in the present study, but might underline the non-NO-mediated vasodilatory pathway. The aortic effects of testosterone need further investigation, in androgen-treated ovariectomized female rats for example, before firm conclusions are made.

The effects of mifepristone were unlikely to have been due to its antagonism of progesterone or glucocorticoid action (Philibert, 1984) in the rat aorta. Progesterone has vasodilatory activity in rat aorta (Glusa et al., 1997; Mukerji et al., 2000; Zhang et al., 2002) but it is not mediated via the nuclear progesterone receptor and thus should be mifepristone insensitive.
Women (Talbott et al., 1997; Selles et al., 2002; Zhang et al., 2002). Mifepristone can also have had minimal acute effect in vitro, as the aortic rings were repeatedly washed (Zhang et al., 2002). A long-term mifepristone effect on aortic gene expression was also unlikely, since 8 days of progesterone injection in ovariectomized rats had no effect on aortic ring vasodilation (Sampaio-Moura and Marcondes, 2001). Finally, glucocorticoids inhibit prostacyclin and NO synthesis in rat aorta (Jeremij and Dondona, 1986; Wallerath et al., 1999), thus mifepristone blockade of glucocorticoid action promotes aortic ring vasodilation in vitro (Selles et al., 2002), in contrast to the present results.

Hyperlipidaemia impairs endothelial function (Shimokowa et al., 1989), but the effect of hyperglycaemia is controversial (Poston and Taylor, 1995; Oltman et al., 1997). Thus changes in aortic function in this study may be the result of metabolic modifications related to mifepristone injection. This model is, however, poorly characterized at the metabolic level and it is difficult to address the possible involvement of metabolic dysfunction at this time.

Mifepristone-injected female rats displayed disrupted ovarian morphology and elevated serum LH and testosteron, as previously seen in this model and in human PCOS women (Ruiz et al., 1996, 1997; Sánchez-Criado et al., 1992, 1993). They provide a valid model of PCOS (Ruiz et al., 1996) in which vascular function can be investigated in greater detail than in humans, using in vitro analysis. Findings in this model will assist the investigation of the association between PCOS and hypertensive disorders of pregnancy. An improved contractile response to PE was noted in aortic rings from mifepristone-treated rats. This may be related to their elevated serum testosterone, as seen in human PCOS women (Glusa et al., 1997; Selles et al., 2002; Zhang et al., 2002; Lakhani et al., 2003). Aortic ring behaviour is modified in vitro in a manner suggestive of depressed endothelial NO release or elevated NO degradation. In compensation, smooth muscle sensitivity to NO is elevated, and a NO-uncoupled, ACh-stimulated relaxation mechanism appears activated, perhaps due to increased prostacyclin or EDHF. Perturbations in in vitro behaviour are compatible with the in vivo changes in mechanical properties, and may result from the endocrine perturbations in this model, perhaps in serum testosterone. Endocrine changes in women with PCOS may therefore cause their disturbed arterial properties. Further studies are necessary to characterize the endocrinological and metabolic changes in the mifepristone rat model and to clarify the mechanisms underlying the altered vascular behaviour.

References