

# Variations in Endothelial Function and Arterial Compliance during the Menstrual Cycle

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Female sex hormones have been implicated in the cardioprotection of premenopausal women. However, the cardiovascular actions of these hormones and the effects of their natural fluctuations during the menstrual cycle are not fully understood. We studied changes in vascular function during the menstrual cycle in 15 healthy premenopausal women. Four noninvasive procedures were performed during the early follicular (EF), late follicular (LF), early luteal (EL), and late luteal (LL) phases: flow-mediated dilatation (FMD) of the brachial artery during reactive hyperemia, laser Doppler velocimetry (LDV) with direct current iontophoresis of acetylcholine (ACh) and nitroprusside, whole body arterial compliance (WBAC), and pulse wave velocity. Hormone levels were consistent with predicted cycle phase and showed that all subjects ovulated during the cycle studied. FMD, LDV with ACh, and WBAC varied cyclically, with significant increases from the F to LF phase, sharp falls in the EL phase, and significant recoveries in the LL phase. These changes were most marked

for FMD [EF,  $8.8 \pm 0.6\%$  (mean  $\pm$  SEM); LF,  $10.0 \pm 0.7$ ; EL,  $4.2 \pm 0.6$ ; LL,  $8.6 \pm 0.9$ ] and the LDV response to ACh (EF,  $2.7 \pm 0.2$  V/min; LF,  $3.3 \pm 0.4$ ; EL,  $1.8 \pm 0.3$ ; LL,  $2.7 \pm 0.4$ ). WBAC changed similarly (EF,  $0.58 \pm 0.08$  arbitrary units; LF,  $0.84 \pm 0.06$ ; EL,  $0.65 \pm 0.05$ ; LL,  $0.68 \pm 0.06$ ). Sodium nitroprusside-induced vasodilatation decreased significantly from EF to EL, with no other significant difference, and pulse wave velocity did not vary significantly over the four time points.

Conductance and resistance artery endothelial reactivity and smooth muscle sensitivity to nitric oxide and arterial compliance are modulated significantly in response to the changing hormonal patterns of the menstrual cycle. These findings emphasize the importance of menstrual phase in the interpretation of data on endothelial function and may provide insights into the mechanisms underlying sex differences in cardiovascular risk and other disease processes in premenopausal women. (*J Clin Endocrinol Metab* 86: 5389–5395, 2001)

IT IS WELL established that the incidence of coronary heart disease (CHD) in women at all ages is lower than that in men and increases after menopause, when ovarian secretion of sex hormones is low (1, 2). There is evidence that postmenopausal E-containing hormonal therapy reduces CHD risk (3), suggesting that ovarian hormones may provide protection against CHD in premenopausal women. This is supported by the known actions of female sex hormones on the cardiovascular system, which include effects on plasma lipid levels (4) and direct (5, 6) and indirect (7, 8) actions on vessel wall physiology. In addition, the natural hormonal fluctuations during the menstrual cycle are reflected in cardiovascular changes. For example, total plasma cholesterol, low density lipoprotein cholesterol, and apolipoprotein A1 concentrations decrease and high density lipoprotein cholesterol and apolipoprotein (a) increase during the luteal phase compared with the follicular phase (9–11), and many circulating factors, such as nitric oxide synthase (12), vascular endothelial growth factor (13), and P-selectin (14), demonstrate cyclical variation. It has also been shown that decreases occur in large vessel endothelial function (15) and radial arterial distensibility (16) in the late luteal phase and in cutaneous

vasodilatation in the menstrual compared with the follicular phase (17). However, there have been no studies that have documented in detail the changes occurring simultaneously in large and small vessel endothelial function, nonendothelial smooth muscle function, and arterial elasticity or compliance both within and between follicular and luteal phases of the normal menstrual cycle.

A detailed understanding of variations in cardiovascular function during the menstrual cycle is of importance for three reasons: it may assist with the interpretation of vascular parameters in premenopausal women; it may provide insights into the mechanisms underlying sex differences in cardiovascular risk; and it may assist in the early detection of cardiovascular disease. We have therefore investigated changes in vascular function using established noninvasive techniques at four time points during the menstrual cycle in healthy young women. The techniques employed allow assessment of small and large vessel endothelial function, small vessel vascular smooth muscle function, and arterial compliance.

## Subjects and Methods

### Subjects

Fifteen healthy, normotensive, nondiabetic, nonsmoking women, aged 19–31 yr, with a body mass index less than  $25 \text{ kg/m}^2$ , with regular menstrual cycles, and not taking any form of oral contraception, were recruited from the general community. The study was approved by the

Abbreviations: ACh, Acetylcholine; CHD, coronary heart disease; EF, early follicular; EL, early luteal; FMD, flow-mediated dilatation; LF, late follicular; LDV, laser Doppler velocimetry; LL, late luteal; PWV, pulse wave velocity; SNP, sodium nitroprusside; WBAC, whole body arterial compliance.

ethics committee of the Alfred Hospital, and all subjects gave written informed consent.

### Study design

Four noninvasive procedures were performed to investigate possible variations in conductance and resistance artery endothelial and smooth muscle function and arterial compliance during the menstrual cycle: flow-mediated dilatation (FMD), laser Doppler velocimetry with direct current iontophoresis (LDV), whole body arterial compliance (WBAC), and pulse wave velocity (PWV). Each subject was studied at four points during the cycle, corresponding to early follicular (EF;  $d 3 \pm 3$ ), late follicular (LF;  $d 12 \pm 3$ ), early luteal (EL;  $d 20 \pm 3$ ), and late luteal (LL;  $d 28 \pm 3$ ) phases. These phases were determined on the basis of previous cycle length and the time of menstruation, using the assumption that luteal phase duration was 14 d. Data were analyzed by the operator who performed the procedures after coding and blinding the operator to the phase of the menstrual cycle.

### FMD

FMD, a noninvasive technique to assess endothelium-dependent and -independent vasorelaxation in a medium-sized artery, was assessed in accordance with established protocols (15, 18). A high resolution ultrasound transducer was placed over the brachial artery to measure its diameter before, during, and after reactive hyperemia. Briefly, after a 15- to 20-min rest period, the right brachial artery was scanned using a 7.5-Hz linear array transducer over a longitudinal section 5–7 cm above the right elbow. A blood pressure cuff around the forearm distal to the target area was inflated to a pressure of 250 mm Hg for 4.5 min and then abruptly deflated, after which a second scan was performed continuously for 90 sec. After a further 10 min of rest, a final scan was performed over the same area. The ultrasound images were recorded on video-cassette. The diameter of the brachial artery was measured from the tunica intima at a fixed distance from the chosen marker. The mean diameters of the brachial artery before, during, and after reactive hyperemia were calculated from three cardiac cycles synchronized with the R-wave peaks on the electrocardiogram. To assess endothelium-independent vasodilation of the brachial artery, the same ultrasound scanning protocol was used after the administration of sublingual glyceryl trinitrate (300  $\mu$ g).

The technique was assessed for reproducibility by measuring the brachial artery diameter and percent change in diameter after reactive hyperemia in six normal subjects (male and postmenopausal female). Each subject was measured on six occasions over 3 consecutive d, and the variability of these measurements was determined. The coefficient of variation for the brachial artery measurements was  $0.34 \pm 0.03$ , and that for the percent change after reactive hyperemia was  $0.45 \pm 0.05\%$ . Measurements were taken from super-VHS video tape recordings by a single observer.

### LDV with direct current iontophoresis

LDV with direct current iontophoresis is a noninvasive approach that permits examination of vascular reactivity in cutaneous arterioles. Blood flow responses to the endothelial vasodilator acetylcholine (ACh) and the smooth muscle-mediated nonendothelial dilator sodium nitroprusside (SNP) were measured using LDV with direct current iontophoresis. LDV uses the Doppler shift of the reflection of a low energy laser beam from moving erythrocytes to quantify microvascular perfusion (19). Iontophoresis uses an electrical direct current as a means of introducing charged substances into the skin.

LDV was performed as previously described (20). Briefly, blood flow was measured with a dual channel Moor DT4 laser Doppler flowmeter (Moor Instruments, Devon, UK) that employs an 810-nm probe to detect blood flow in the superficial 1–2 mm of skin. A continuous tracing of blood flux was made using a chart recorder (21). Drugs were iontophoresed from specially designed polyvinylchloride chambers containing a reservoir about 0.5 ml in capacity and a central well 6 mm in diameter applied to the forearm with double-sided adhesive tape. The solutions used were ACh (BDH Chemicals, Poole, UK) and SNP (David Bull Laboratories, Inc., Mulgrave, Australia), each at a concentration of 10 mg/ml. A current of 0.04 mA was passed for 30 sec through a circular

chamber 8 mm in diameter, giving a charge density of 0.08 mA/cm<sup>2</sup>. SNP was administered with a cathodal charge, and ACh with an anodal charge. Subjects rested for 20 min before testing to establish a stable baseline. Blood flux was measured continuously for 4 min after completion of stimulus for ACh and for 6 min for SNP. The responses were quantified by measuring the area under the curve of each response, as recorded on the chart recorder (19). The coefficient of variation for blood flux assessed using this method is  $0.15 \pm 0.02$ , as tested in a cohort of postmenopausal women.

### WBAC and PWV

Arterial compliance and PWV were measured as markers of arterial elasticity. WBAC was based on a two-element Windkessel model of the circulation and area method of Liu *et al.* (22) by simultaneous measurement of ascending aortic blood flow and carotid blood pressure. With the subject lying in the supine position with the head tilted back, aortic flow velocity was measured using a hand-held Doppler flow velocimeter (multidoplex MDI, Huntleigh Technology, Cardiff, UK) placed at the suprasternal notch, and aortic root driving pressure was measured by applanation tonometry of the proximal right carotid artery using a noninvasive Millar Mikro-Tip pressure transducer (model SPT-301, Miller Instruments, Houston, TX). Aortic annular diameter at the base of the aortic valve was measured at peak systole by two-dimensional echocardiography (model SONOS 1500, Phased Array Sector Scanner, Hewlett-Packard Co., Andover, MA). From this measurement, left ventricular outflow tract area was calculated and used to convert velocity to flow volume. Brachial mean and diastolic artery pressures were measured simultaneously using a Dinamap vital signs monitor (1846SX, Critikon, Tampa, FL) and used to calibrate the carotid wave form as described previously (23).

WBAC was calculated using purpose-written software (Dr. J. Cameron, Alfred Hospital, Australia). For each subject, at each time interval, the average WBAC was determined by analyzing at least 10 sets of flow and pressure wave forms. PWV was determined by simultaneous applanation tonometry of the carotid and the femoral arteries and calculation of transit velocity by dividing by the distance between the two sites. To prevent bias from pulse pressure wave reflections, measurements were taken at the beginning of the systolic upstroke (23). For each subject at each time interval, PWV was determined by analyzing all of the pressure wave forms that were acquired during the 10-sec measuring period.

The techniques were assessed for reproducibility in 50 healthy volunteers (males and postmenopausal females). Each subject was measured on 2 occasions, and the variability of these measurements was determined. Coefficients of variation were 9.2% and 3.2% for WBAC and PWV, respectively (24).

### Biochemical analysis

At each time point a 20-ml fasting blood sample was taken for measurement of E2, FSH, LH, and progesterone to confirm cycle phase, and total plasma cholesterol and triglycerides. Blood was collected in EDTA tubes and spun at 3000 rpm within 15 min of collection.

Assays of E2, FSH, and LH used ovion kits; E2 was determined by a coated tube RIA; FSH and LH were measured by an immunoradiometric assay. Progesterone assays employed a two-antibody in-house RIA designed and validated at the Baker Medical Research Institute (Melbourne, Australia) that uses [<sup>3</sup>H]progesterone as tracer after preliminary extractions of samples with hexane/ethyl acetate (100:1). Total cholesterol and triglycerides were determined enzymatically with an Ek-tachem Kodak diagnostic system (Rochester, NY).

### Statistical analysis

All data are presented as the mean  $\pm$  SEM. Comparisons were performed pairwise using one-way ANOVA with repeated measures and least significant difference tests. The null hypothesis was rejected at  $P < 0.05$ . Where multiple comparisons were made, a Bonferroni correction was applied.

**TABLE 1.** Clinical characteristics of subjects and plasma hormone levels

Characteristics	EF	LF	EL	LL
Total cholesterol (mmol/liter)	5.2 ± 0.3	5.2 ± 0.3	5.3 ± 0.3	5 ± 0.3
Triglycerides (mmol/liter)	1.1 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2
Systolic blood pressure (mm Hg)	109 ± 3	104 ± 3	100 ± 3	103 ± 5
Diastolic blood pressure (mm Hg)	65.8 ± 1.1	63.5 ± 2.2	63.8 ± 1.8	64.8 ± 2
Mean arterial pressure (mm Hg)	82 ± 2	77 ± 2	78 ± 2	81 ± 3
Serum E2 (pmol/liter)	94 ± 10.15 <sup>a</sup>	378 ± 78	368 ± 51	326 ± 44
Serum progesterone (nmol/liter)	0.2 ± 0.0 <sup>b</sup>	0.3 ± 0.1 <sup>b</sup>	5.3 ± 2	8.2 ± 1
Serum FSH (IU/liter)	6 ± 0.3	6 ± 0.3	4 ± 0.4 <sup>c</sup>	3 ± 0.4 <sup>a</sup>
Serum LH (IU/liter)	4 ± 0.6 <sup>b</sup>	10 ± 2	11 ± 3	4 ± 0.9 <sup>b</sup>

<sup>a</sup>  $P < 0.01$  compared with all other phases.<sup>b</sup>  $P < 0.001$  compared with the two highest values.<sup>c</sup>  $P < 0.05$  compared with highest value.

## Results

The mean age of the women was  $22.8 \pm 0.7$  yr. All women were nulliparous, and the mean length of their cycles was  $29 \pm 5$  d. Hormonal, lipid, and blood pressure measurements for each time point are shown in Table 1. E2, progesterone, LH, and FSH levels were consistent with the predicted cycle phase, and individual progesterone measurements showed that every subject ovulated during the cycle studied. Pre-ovulatory surges in FSH and LH were not observed; however, a significant increase in LH was observed at the time of ovulation, and a significant decrease in FSH was observed during the EL and LL phases ( $P < 0.05$ ). Progesterone levels were significantly higher in the EL compared with the LF phase ( $P < 0.001$ ), confirming postovulatory status, although E2 levels were not significantly different. Total cholesterol, triglycerides, systolic and diastolic blood pressures, and mean arterial pressure did not vary significantly during the four phases of the menstrual cycle.

## FMD

Individual and mean values for FMD at the four time points are shown in Fig. 1. Pairwise comparisons showed that EF values were significantly lower than LF values ( $8.8 \pm 0.6\%$  vs.  $10 \pm 0.7\%$ , respectively), and EL values were significantly lower than LL ones ( $4.2 \pm 0.6\%$  vs.  $8.6 \pm 0.9\%$ ). Early luteal values were significantly different from those in each of the other three phases. Mean baseline brachial artery diameters were 0.29, 0.29, 0.29, and 0.28 cm in the EF, LF, EL, and LL phases, respectively; there was no significant difference between the various time points. There were no significant changes in brachial artery vasodilation at any of the four points in response to glyceryl trinitrate ( $8.6 \pm 1.7\%$ ,  $8.7 \pm 1.4\%$ ,  $9.6 \pm 0.5\%$ , and  $8.1 \pm 1.1\%$ ).

## LDV with direct current iontophoresis

Individual and mean blood flux responses to ACh and SNP are shown in Fig. 2. ACh-induced vasodilation was significantly lower ( $P < 0.05$ ) in the EF phase than in the LP phase ( $2.7 \pm 0.2$  vs.  $3.3 \pm 0.4$  V/min), and responses in the EL phase were significantly lower ( $P < 0.05$ ) than those in the LL phase ( $1.8 \pm 0.3$  vs.  $2.7 \pm 0.4$  V/min). SNP-induced vasodilatation was significantly greater in the EF phase than in the EL phase ( $4.8 \pm 1.1$  vs.  $2.4 \pm 0.7$  V/min), but other differences failed to reach significance.

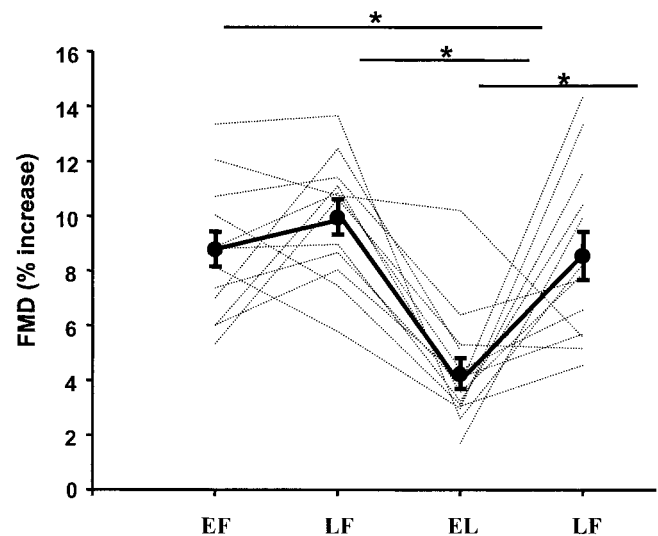


FIG. 1. A, FMD of the brachial artery with reactive hyperemia across the four phases of the menstrual cycle. Individual data are shown together with the mean ± SEM. \*, Significant difference from one phase to another ( $P < 0.05$ ). Endothelium-independent changes in brachial artery vasodilation did not significantly change across the four phases of the menstrual cycle (data shown in text).

## WBAC and PWV

Individual and mean values for WBAC and PWV are presented in Fig. 3. Due to difficulties in data analysis (relating to imperfect wave forms unable to be processed by the analysis program), three subjects were not included in the analysis of WBAC. WBAC was significantly greater ( $P < 0.05$ ) during the LF than in the EF and EL phases ( $0.8 \pm 0.1$  vs.  $0.6 \pm 0.1$  and  $0.7 \pm 0.0$  systemic arterial compliance arbitrary units, respectively). There were no significant differences between values obtained in EF, EL, and LL phases. Central PWV did not vary significantly over the four time points.

## Discussion

This study shows that in healthy young women during normal ovulatory menstrual cycles, FMD, cutaneous vasodilatory responses to iontophoresed ACh and SNP, and WBAC all change in a cyclical fashion. Specifically, brachial artery FMD and cutaneous ACh-induced dilatation (markers of endothelium-dependent vasodilation in conductance and resistance arteries) increase from the menstrual to the LF phase, fall in the EL phase, and increase again in the LL

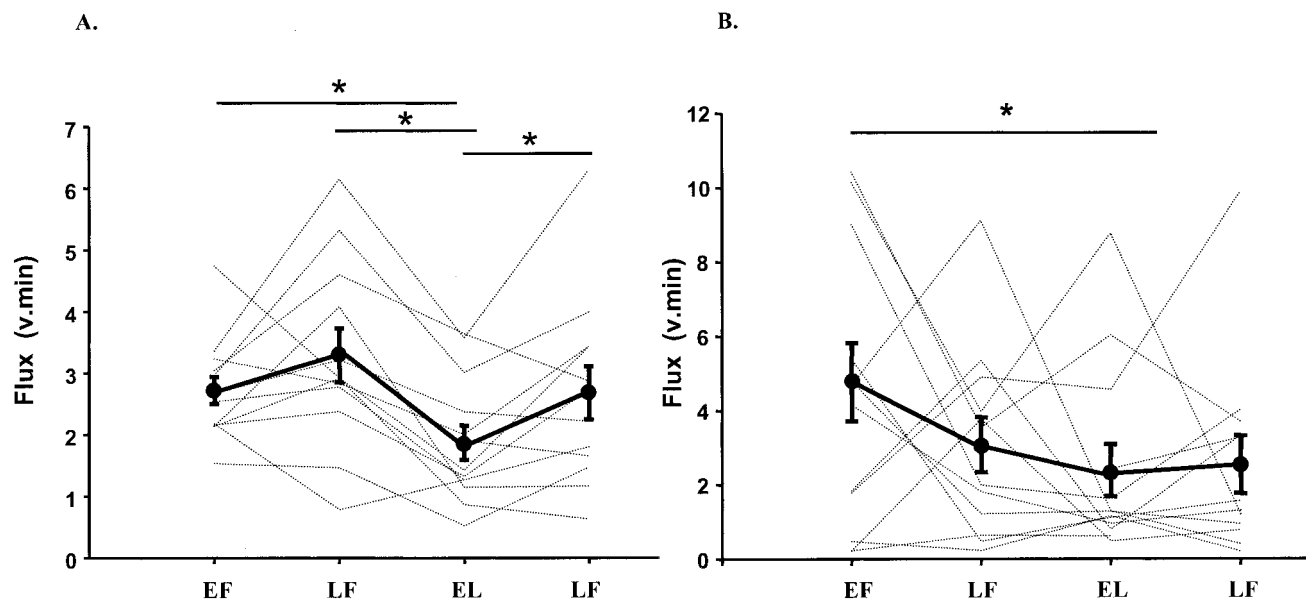


FIG. 2. A, Cutaneous blood flux measured by LDV of ACh in response to direct current iontophoresis across the four phases of the menstrual cycle. B, Cutaneous blood flux in response to iontophoretically applied SNP. \*, Significant difference between phases ( $P < 0.05$ ).

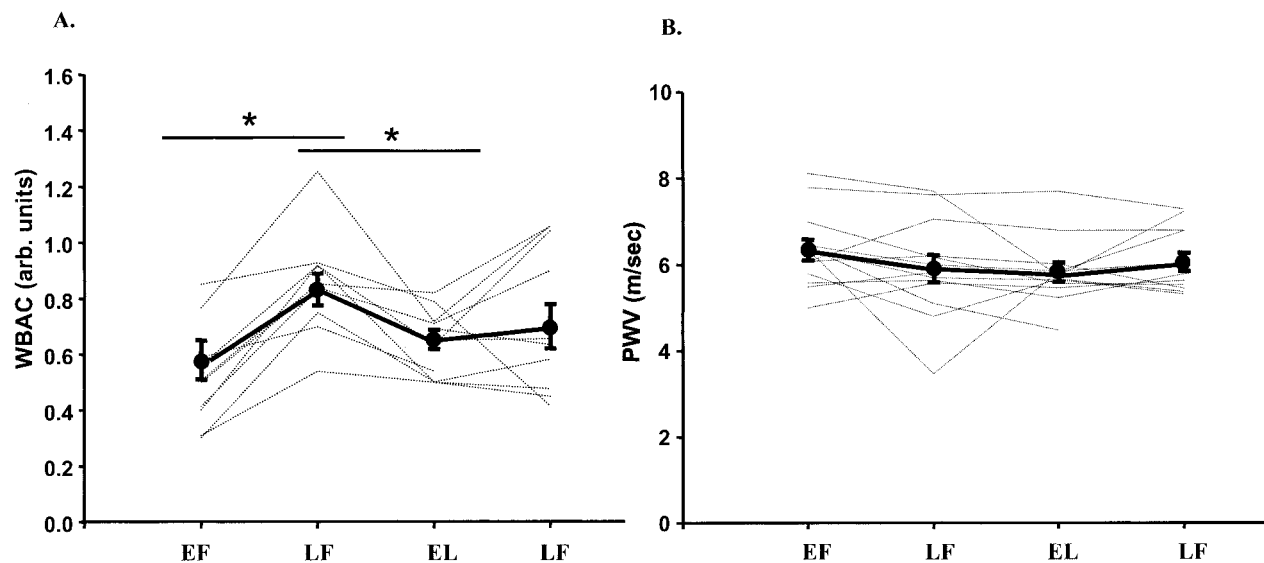


FIG. 3. A, WBAC across the phases of the menstrual cycle. \*, Significant difference between phases of the menstrual cycle ( $P < 0.05$ ). B, PWV across the phases of the menstrual cycle.

phase, whereas WBAC increases from the menstrual to the LP phase and falls in the EL phase, and SNP-induced dilatation falls from the menstrual to EL phase. By contrast, PWV and brachial artery endothelium-independent vasodilation do not appear to change during the menstrual cycle, and in this study, there were no observable changes in lipid levels or blood pressure.

Vascular tone has been shown to be influenced by a variety of neural and humoral factors. FMD in large conduit vessels is augmented by increased synthesis and release of nitric oxide from the endothelium of large and small vessels and is reduced by inhibition of nitric oxide synthase by  $N^G$ -monomethyl-L-arginine (25). ACh-induced vasodilatation reflects the release of nitric oxide from the endothelium, with a possible contribution from PG production (26). SNP-

induced vasodilation is thought to be due to direct effects on vascular smooth muscle, apparently independent of the endothelium. The effects of iontophoretically applied ACh and SNP are restricted to the cutaneous microvasculature and do not appear to involve sensory nerve activation (27) or activation of nicotinic receptors (20). Accordingly, the present study suggests that the changing hormonal milieu during the menstrual cycle is associated with changes in large vessel and microvascular endothelial and smooth muscle function.

WBAC gives a measure of both central and peripheral compliance, whereas PWV is a measure of regional compliance, excluding both the peripheral component and that involving the aortic arch. It is known that arterial compliance decreases with aging and menopause and in disease states such as atherosclerosis and hypertension and increases with

postmenopausal E treatment (28, 29). As with FMD and ACh, our results show improvements in WBAC during follicular development and a fall after ovulation, although in this case there was no further significant change during the luteal phase of the cycle. In contrast, central PWV did not vary between menstrual phases. It is possible that these differences in the findings for WBAC and PWV reflect the differences in the vascular beds from which they are derived. For example, the aortic arch is known to contain functional ERs (30), and E can act directly on the vasa vasorum in the peripheral vasculature (31), as a result of either of which WBAC could change independently of PWV in response to varying hormone levels. In other studies it has been shown that arterial compliance can vary with changes in blood pressure (32) and lipid levels (33), but these do not appear to be relevant factors here. Accordingly, we conclude that the changes observed in WBAC most likely reflect cyclical effects on the peripheral vasculature and/or directly on the aortic arch.

Our results are broadly consistent with those obtained in previous studies. It has been shown, for example, that in healthy young women, FMD is higher in the follicular phase than in either luteal or menstrual phases (15, 34), that radial arterial distensibility, measured as the ratio of radial artery diameter to blood pressure, is increased in the ovulatory compared with the luteal phase (35), that in postmenopausal women E2 therapy leads to an increase in FMD that is not attenuated by the addition of micronized progesterone (36), and that the cutaneous response to ACh is greater at midcycle than during menses (37). One study using ultrasonic wall tracking and measurement of brachial artery cuff pressure reported no changes in carotid artery distensibility and cross-sectional compliance during the phases of the menstrual cycle (38); the discrepancy between these findings and our own probably relate to methodological differences, in particular the use of peripheral brachial pressures, which may vary considerably from true central pressures in the region of the carotid artery (39). The present study extends the results of these previous studies to document in detail the precise time course and parallel nature of the vascular changes that occur during the menstrual cycle. It also draws particular attention to the importance of the sharp drop in markers of both large and small vessel endothelial function immediately after ovulation. In addition, it shows for the first time that arterial compliance undergoes similar changes, whereas PWV, a measure of central compliance, does not.

It is likely that the changes demonstrated reflect in part variations in hormonal levels during the menstrual cycle. These include changes in levels of E, which rise during the proliferative phase, fall during early luteal development, and rise slightly during the remainder of the cycle, and in progesterone levels, which remain low during the first half of the cycle and then rise during the LL phase (40). In this study although mean E2 values at the LF and EL time points were similar, the rise in progesterone levels indicates a reduction in E action, supporting a role for this hormone in the changes between the two phases; in addition, the lack of a fall in any of the end points in the late luteal phase suggests that there are no obvious antago-

nizing effects of progesterone *per se*. Nonetheless, no simple relationship can be postulated between levels of either hormone and particular vascular end points. Indeed, it is important to recognize that many other factors affecting the cardiovascular system also vary during the cycle, including nitric oxide (41) and nitric oxide synthase (42), vascular endothelial growth factor (43), prostanoid metabolites (44), adhesion molecules including integrins (45) and P-selectin (46), and homocysteine (47). It is probable that the changes observed in each of the variables assessed in this study are the outcome of complex combinations of factors secondary to the hormonal cycle.

The findings of this study may be of value for the understanding of normal physiology, for elucidating the mechanisms underlying sex differences in cardiovascular risk, and for identifying and investigating specific disease processes. It is clear from our findings that where measurements of large or small vessel endothelial function or of arterial compliance are undertaken in premenopausal women they should be standardized to menstrual phase. In addition, certain disease states are known to be associated with endothelial dysfunction that varies during the menstrual cycle; these include endometriosis and adenomyosis (48), preeclampsia (49), and possibly polycystic ovary disease (50, 51), although here the data are conflicting (52). It is possible that in these cases the changes in the dynamic profile of the vascular parameters themselves have pathological significance. Further, the fact that women with prolonged menstrual irregularity are at increased risk of developing cardiovascular disease later in life (53) suggests that the lower cardiovascular risk well recognized in healthy young women compared with men may reflect not merely differences in hormonal levels, but also the dynamic fluctuations that occur during the menstrual cycle.

The findings of this study are consistent with results obtained from other studies investigating endothelial function and arterial compliance in age-matched male subjects. We have previously reported (20) that mean resting LDV levels in healthy young men are  $1.8 \pm 0.4$ , equivalent to those in the EL phase of the menstrual cycle in this study. Toikka *et al.* (54) showed that FMD in healthy young men was  $5.5 \pm 3.2\%$ , intermediate between EL and LL values in this study, and Liang *et al.* (55) found normal values for arterial compliance in healthy men to be  $0.48 \pm 0.06$  arbitrary compliance units, somewhat lower than the nadir recorded during the menstrual cycle. These comparisons indicate that in women all three vascular function variables are generally higher than those in men, although there is some overlap in the endothelial variables, suggesting that both absolute levels and the temporal profile may be important considerations in cardiovascular risk.

In conclusion, this study shows that in healthy young women during ovulatory menstrual cycles, large and small vessel endothelial function increase during the follicular phase, fall after ovulation and then rise again during the luteal phase of the menstrual cycle, whereas arterial compliance increases during follicular development and falls after ovulation, and nonendothelial smooth muscle function declines from the menstrual to the EL phase. These findings are likely to reflect hormonal fluctuations

either directly or indirectly and suggest that care should be taken in interpreting assessments of cardiovascular disease risk in premenopausal women at a particular time point; their significance for the understanding of disease processes remains to be elucidated.

### Acknowledgments

Received February 12, 2001. Accepted July 30, 2001.

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This work was supported by the Victorian Health Promotion Foundation (to P.K.).

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