The effect of oral contraceptives on the nitric oxide system and renal function

D. Z. I. Cherney,1 J. W. Scholey,1 D. C. Cattran,1 A. K. Kang,1 J. Zimpelmann,2 C. Kennedy,2 V. Lai,1 K. D. Burns,2 and J. A. Miller1

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Cherney DZ, Scholey JW, Cattran DC, Kang AK, Zimpelmann J, Kennedy C, Lai V, Burns KD, Miller JA. The effect of oral contraceptives on the nitric oxide system and renal function. Am J Physiol Renal Physiol 293:F1539–F1544, 2007. First published August 22, 2007; doi:10.1152/ajprenal.00351.2007.—We have demonstrated that oral contraceptive (OC) users exhibit elevated angiotensin II levels and angiotensin II type 1 receptor expression, indicative of renin-angiotensin system (RAS) activation, yet the renal and systemic consequences are minimal, suggesting that there is increased vasodilatory activity, counteracting the effect of RAS activation. We hypothesized that the nitric oxide (NO) system would be upregulated in OC users and that this would be reflected by a blunted hemodynamic response to l-arginine infusion. All subjects were studied after a 7-day controlled sodium and protein diet. Inulin and para-aminohippurate clearance techniques were used to assess renal function. L-Arginine was infused at 100, 250, and 500 mg/kg, each over 30 min. Skin endothelial NO synthase mRNA expression was assessed by real-time PCR. While OC nonusers exhibited significant increases in effective renal plasma flow (670.8 ± 35.6 to 816.2 ± 59.7 ml/min·1.73 m−2) and glomerular filtration rate (133.4 ± 4.3 to 150.1 ± 5.7 ml/min·1.73 m−2, P = 0.04) and declines in renal vascular resistance (81.1 ± 6.1 to 63.5 ± 6.2 mmHg·ml·1·min−1·m−2) at the lower l-arginine infusion rates, the responses in OC users were blunted. While l-arginine reduced mean arterial pressure at the 250 and 500 mg/kg doses in OC nonusers, OC users only exhibited a decrease in mean arterial pressure at the highest infusion rate. In contrast, tissue endothelial NO synthase mRNA levels were higher in the OC users (P = 0.04). In summary, these findings suggest that the NO system is upregulated by OC use in young, healthy women. Increased activity of the NO pathway may modulate the hemodynamic effects of RAS activation in OC users.

renin-angiotensin system; renal hemodynamic function

We have demonstrated in women who are users of oral contraceptive (OC) medications, that angiotensin (ANG) II levels are dramatically increased (23), but renal and systemic changes are minimal (9, 22, 23). Furthermore, we found that skin ANG II type 1 receptor (AT1R) mRNA was increased in OC users and that the hemodynamic response to ANG II was augmented, suggesting that AT1R homologous downregulation cannot be the mechanism responsible for the maintenance of normal hemodynamic function. Although the mechanisms that defend OC users against the hemodynamic effects of ANG II are not known, a functional feedback balance has been postulated between the nitric oxide (NO) pathway and the renin-angiotensin system (RAS) (42). Estrogen-mediated activation of the NO system may be an important factor that blunts the hemodynamic effect of RAS activation.

Experimental evidence suggests that estrogen alters vascular function by enhancing NO production by the vascular endothelium (29). Physiological levels of estrogen cause a rapid release of NO in cultured bovine and human endothelial cells (2, 6) and enhance endothelial-dependent vasodilatation (7). These effects are mediated by increased transcription of endothelial NO synthase (eNOS) (25, 44) and by Akt-dependent activity of eNOS (12). Estrogen may also increase the vascular responsiveness to NO donors by enhancing NO bioavailability, possibly through antioxidant mechanisms (3, 15, 54). Considering these well-known genomic and nongenomic effects of estrogen on the NO pathway (7, 18), it is conceivable that NO is an important modulator of the hemodynamic effects of OC-induced RAS activation.

The overall objective of this set of experiments was to examine the mechanism whereby women who are OC users maintain normal renal and systemic hemodynamic function in the face of RAS activation. We hypothesized that the hemodynamic response to a graded l-arginine infusion would differ in normal, healthy women who were users and nonusers of OCs, in that users would exhibit a blunted renal and systemic hemodynamic response to l-arginine infusion, compared with OC nonusers. OC nonusers were studied during the follicular phase of the menstrual cycle when estrogen levels are low to amplify the hormonal differences between the two groups, similar to previous studies from this laboratory (22).

METHODS

Subjects. The study was performed with the approval of the University Health Network Research Ethics Board and with the informed, written consent of each subject. Recruitment was in accordance with the policies of the Human Subjects’ Review Committee of the University of Toronto. We studied age-matched, otherwise healthy women, aged 18–40 yr. All subjects had normal values for body mass index, arterial pressure, renal function, liver function, and electrocardiogram. Exclusion criteria included a history of renal, cardiac, or lung disease, or current smoking. Except for OCs, no subjects ingested any regular medications. Pregnancy was excluded with a negative serum β-human chorionic gonadotropin test before enrollment. All subjects were interviewed and examined by a qualified internist.

Preparation. Each subject was studied on one occasion. OC users were studied during the first 21 days of the menstrual cycle, and OC nonusers were studied during the first 7 days of the menstrual cycle. As in previous protocols from these investigators (8, 9, 22, 40), each study was performed after 7 days on a controlled diet consisting of 150 mmol/day sodium and 1.5 g·kg−1·day−1 protein. Compliance was ascertained by measurement of 24-h urine sodium, potassium, and urea excretion on the 7th day. Data were analyzed if urine sodium excretion were >150 mmol/day and urea excretion were 3–6

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mmol·kg⁻¹·day⁻¹. No subjects were excluded. Each subject presented to the Renal Physiology Laboratory at the Toronto General Hospital at 0800 after an overnight fast.

**Experimental procedures.** Each subject had an 18-gauge peripheral venous cannula inserted into an antecubital vein for sampling and a second cannula in the contralateral arm for infusions. Each OC nonuser had a blood sample collected for estradiol levels, to delineate phases of the menstrual cycle. Data were not used if levels were inappropriate to the follicular phase. No subjects were excluded based on those criteria. Blood samples were collected for inulin blank and for baseline values for renin, plasma renin activity (PRA), ANG II, aldosterone, and cGMP. Hemodynamic parameters (arterial pressure, heart rate) were measured by an automated sphygmonanometer (Dinamap, Critikon) at 15-min intervals throughout the study. Renal hemodynamic function was assessed using inulin and para-aminomipipurate (PAH) clearance techniques, as previously described in this investigator’s studies (8, 9, 22, 40). The inulin and PAH clearances, corrected for body surface area, represented glomerular filtration rate (GFR) and estimated renal plasma flow (ERPF), respectively, expressed per 1.73 m². Filtration fraction (FF) was determined by dividing the GFR by the ERPF. Renal blood flow (RBF) was calculated by dividing the ERPF by (1 − hematocrit). Renal vascular resistance (RVR) was derived by dividing the mean arterial pressure (MAP) by the ERBF. After two clearance periods had been completed, L-arginine (Clinalfa) was infused at 100, 250, and 500 mg/kg, with each infusion period over 30 min. Renal clearances were repeated after each infusion period and again after a 30-min recovery period was complete.

ANG II was measured by radioimmunoassay. Blood was collected into prechilled tubes containing EDTA and angiotensinase inhibitor (0.1 ml Bestatin Solution, Buhlmann Laboratories). After centrifugation, plasma samples were stored at −70°C until analysis. On the day of analysis, plasma samples were extracted on phenylisilicila columns. A competitive radioimmunoassay kit supplied by Buhlmann Laboratories was used to measure the extracted ANG II. Aldosterone was measured by radioimmunoassay, using the Coat-A-Count system. Angiotensinogen was measured indirectly by converting endogenous angiotensinogen to ANG I and then quantitating the amount of ANG I by radioimmunoassay (PRA). Conversion was done by incubating the plasma with an excess amount of exogenous renin at 37°C for 18 h. After measuring the produced ANG I, the endogenous ANG I obtained before incubation was subtracted (50). Active plasma renin was measured by two-site immunoradiometric assay, where two monoclonal antibodies to human active renin are used. One antibody was coupled to biotin, while the second was radiolabeled for detection. The sample containing active renin was incubated simultaneously with both antibodies to form a complex. The radioactivity of this complex was directly proportional to the amount of immunoreactive renin present in the sample (52). Plasma cGMP samples were deproteinized with ethanol. cGMP was measured using the acetylation method with an assay kit purchased from Cayman Chemical (Ann Arbor, MI).

A skin biopsy was obtained from each subject under sterile conditions after subcutaneous infusion of local anesthetic with xylocaine. In OC nonusers, the biopsy was obtained during the follicular phase. Skin biopsies were stored in liquid nitrogen at −70°C before processing. eNOS mRNA levels were assessed by a PCR protocol. Reverse transcription-PCR was performed using the One-Step RT-PCR kit (Applied Biosystems, Foster City, CA). One-step PCR was performed under the following conditions: 48°C for 30 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s, using a real-time PCR protocol and the ABI PRISM 7000 Sequence Detection System. Real-time PCR was performed using a One-Step RT-PCR kit from Applied Biosystems. RNA (25–50 ng) was isolated from skin samples using an RNAeasy kit (Qiagen, Chatsworth, CA). The primers were as follows: human eNOS forward primer, CGC AGC GCC GTG AAG (9796–9806); human eNOS reverse primer, ACC ACG TCA TAC TCA TCC ATA CAC (10181–10204); human eNOS probe, CCT CGC TCA TGG GCA CGA TTG (9814–9833). The final eNOS primer concentrations were 0.5 μM, and the eNOS probe concentration was 0.2 μM. The eNOS probe uses a FAM dye with a TAMRA quencher. Human GAPDH primers were used at a final concentration of 0.1 μM, and the human GAPDH probe concentration was 0.05 μM. The GAPDH probe uses a JOE dye with a TAMRA quencher. Real-time PCR was carried out using a real-time PCR-ABI PRISM 7000 Sequence Detection System. All data for eNOS expression were corrected for GAPDH expression.

**Analysis.** Data were analyzed using the SPSS (SPSS version 14.0 Graduate Package for Students) computer program. Outcome measures consisted of within-subject changes from baseline in GFR, ERPF, RBF, FF, RVR, ANG II, renin, PRA, aldosterone, cGMP, and MAP and were assessed using repeated-measures analysis of variance. Between-group baseline differences were assessed using nonparametric tests.

**RESULTS**

**OC users vs. nonusers.** At baseline, OC users and nonusers exhibited similar systemic arterial pressure parameters and similar dietary intake of sodium and protein (Table 1). Renal hemodynamic testing revealed that the GFR, ERPF, RBF, FF, and RVR were also similar. At baseline, aldosterone (P = 0.03) and ANG II levels (P = 0.012) were elevated in OC users. PRA, renin, and cGMP levels were not significantly different between the two groups at baseline. At the time of their investigations in OC nonusers, estrogen levels were consistent with the follicular phase of the menstrual cycle.

**Response to L-arginine.** The MAP was not affected by L-arginine in OC users until the highest dose, whereas OC nonusers exhibited significant hemodynamic effects at the lower 250 mg/kg dose (Table 2). While there were no significant changes in renal hemodynamic parameters in the OC group (Table 2), OC nonusers exhibited significant renal hemodynamic responses to L-arginine, in that the GFR and ERPF increased significantly (Figs. 1–3).

Although the rise in cGMP occurred in both groups, OC users exhibited an augmented L-arginine-mediated response, with increased cGMP production at each of the three infusion rates. While aldosterone and ANG II declined in both groups, the decrease was greater in the OC group for aldosterone (P = 0.03) and ANG II (P = 0.012) (Table 3). The declines in ANG II levels were greater in the OC group at 250 and 500 mg/kg (P = 0.01). No significant between-group differences in PRA or renin levels were detected at baseline or in response to L-arginine.

**Skin biopsy eNOS receptor expression.** Data are presented as the ratio of human eNOS receptor mRNA to GAPDH mRNA in arbitrary units. OC users exhibited higher eNOS mRNA expression compared to nonusers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OC User Group</th>
<th>OC Nonuser Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Age, yr</td>
<td>26 ± 5</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>60 ± 10</td>
<td>60 ± 11</td>
</tr>
<tr>
<td>Estrogen, pmol/l</td>
<td>N/A</td>
<td>67 ± 11</td>
</tr>
<tr>
<td>Sodium excretion, mmol/24 h</td>
<td>214 ± 29</td>
<td>185 ± 20</td>
</tr>
<tr>
<td>Protein intake, g·kg⁻¹·day⁻¹</td>
<td>1.2 ± 0.06</td>
<td>1.1 ± 0.05</td>
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Values are means ± SE; n, no. of subjects. OC, oral contraceptive.

Table 1. Baseline characteristics
were that increased activity of the NO system. Our principle findings
our aim was to determine whether OC use was associated with
components, plasma levels of cGMP, and tissue expression of eNOS.
related these responses to changes in circulating RAS com-
ynens, young women: OC users and OC nonusers. In addition, we
infusion in both groups but remained higher in the OC users;
expression corrected for GAPDH signal in skin biopsy speci-
expression in skin biopsy specimens (4.9 ± 0.9 vs. 1.5 ± 0.4, P = 0.04) (Fig. 4).

DISCUSSION

In this study, we examined the renal and systemic hemody-
activities to L-arginine infusion in two groups of healthy
young women: OC users and OC nonusers. In addition, we
related these responses to changes in circulating RAS compo-
nents, plasma levels of cGMP, and tissue expression of eNOS.
Our aim was to determine whether OC use was associated with
increased activity of the NO system. Our principle findings
were that 1) the systemic and renal hemodynamic responses to
arginine infusion were blunted in OC users compared with
OC nonusers; 2) circulating levels of the effectors of the RAS
system, ANG II and aldosterone, declined during L-arginine
infusion in both groups but remained higher in the OC users;
3) baseline tissue expression of eNOS was also augmented in
OC users.

Studies have suggested both activation (11, 19, 48) and
suppression (47) of the RAS in OC users. A promoter region in
the angiotensinogen gene is responsive to estrogen (13), and
exogenous estrogen administration raises plasma, hepatic, and
renal angiotensinogen levels and has the potential to raise
plasma concentrations of ANG II. For example, the ingestion
of ethinyl estradiol as part of a combined OC leads to an
increase in plasma angiotensinogen that is only slightly less
than that seen during pregnancy (38). In previous studies, our
laboratory has observed increases in ANG II, angiotensinogen,
and aldosterone levels in OC users, with only minimal hemo-
dynamic consequences (22), despite increased tissue AT1R
expression (1).

NO is synthesized from the amino acid L-arginine by the
enzyme NO synthase (NOS) (41). In the vascular endothelium,
NO regulates vasodilator tone by activating soluble guanylate
cyclase and increasing intracellular production of cGMP (20).
Although all three types of NOS (eNOS, neuronal NOS,
and inducible NOS) are present in the kidney, only eNOS and
inducible NOS are directly involved in the regulation of ERPF
and GFR under normal physiological conditions (26). The
investigation of the physiological role of NO in regulation of
arterial pressure, ERPF, and GFR has relied on the use of
nonselective NOS inhibitors, such as Nω-monomethyl-L-argin-
ine, and infusions of the physiological precursor of NO,
L-arginine. Previous work has suggested that NO acts as a tonic

| Table 2. Renal and systemic hemodynamic responses to L-arginine in OC users vs. follicular phase subjects |
|--------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                    | Baseline       | 100 mg/kg       | 250 mg/kg       | 500 mg/kg       |
| OCP users                          |                |                 |                 |                 |
| MAP, mmHg                          | 79±2           | 76±3            | 74±2*           | 73±3†           |
| GFR, ml/min⁻¹·1.73 m⁻²              | 133±4          | 136±5           | 142±7           | 151±6§          |
| ERPF, ml/min⁻¹·1.73 m⁻²             | 671±56         | 684±40          | 739±50§         | 816±60§         |
| FF                                 | 0.20±0.01      | 0.20±0.01       | 0.20±0.01       | 0.20±0.01       |
| RBF, ml/min⁻¹·1.73 m⁻²              | 1,104±93       | 1,058±62        | 1,132±77        | 1,227±90        |
| RVR, mmHG·ml⁻¹·min⁻¹·1.73 m⁻²       | 81±6           | 77±6            | 71±7§           | 65±6§           |
| OCP nonusers                        |                |                 |                 |                 |
| MAP, mmHg                          | 80±2           | 78±2            | 77±2            | 75±2‡           |
| GFR, ml/min⁻¹·1.73 m⁻²              | 124±4          | 124±7           | 125±6           | 131±6           |
| ERPF, ml/min⁻¹·1.73 m⁻²             | 645±21         | 641±22          | 678±25          | 720±39          |
| FF                                 | 0.20±0.02      | 0.19±0.01       | 0.19±0.01       | 0.19±0.01       |
| RBF, ml/min⁻¹·1.73 m⁻²              | 1,002±36       | 1,002±42        | 1,047±47        | 1,081±60        |
| RVR, mmHG·ml⁻¹·min⁻¹·1.73 m⁻²       | 85±4           | 84±4            | 80±4            | 76±6            |

Values are means ± SE. OCP, OC phase; MAP, mean arterial pressure; GFR, glomerular filtration rate; ERPF, effective renal plasma flow; FF, filtration fraction; RBF, renal blood flow; RVR, renal vascular resistance. In OC nonusers, *P = 0.005 at 250 mg/kg and †P = 0.046 at 500 mg/kg vs. baseline MAP values. In OC users, ‡P = 0.006 at 500 mg/kg vs. baseline MAP values. In OC nonusers, §P < 0.05 vs. baseline for renal hemodynamic parameters.

Fig. 1. Effective renal plasma flow (ERPF) in oral contraceptive (OC) users and nonusers. The ERPF response to L-arginine is shown. OCP, OC phase. *P = 0.011, †P = 0.002, ‡P = 0.001, and §P = 0.001 vs. baseline ERPF.

Fig. 2. Glomerular filtration rate (GFR) in OC users and nonusers. The GFR response to L-arginine is shown. *P = 0.04 vs. baseline GFR.
vasodilator regulating basal renal and systemic vascular hemodynamics and is important in arterial pressure regulation (16). Infusion of L-arginine results in a rise in ERPF and GFR in control animals (24) and in normal humans (4, 28, 45).

Our first major observation was that L-arginine infusion led to a rise in ERPF and a decline in RVR in OC nonusers. We also observed a rise in GFR, which, in the context of a decline in RVR and a rise in ERPF, suggests that the predominant effect of L-arginine infusion is to cause relatively more afferent than efferent vasodilatation. In OC users, these responses were significantly blunted. We believe that the blunted response to L-arginine infusion supports the hypothesis that there is increased activity of the NO system in OC users, and that further delivery of the substrate for eNOS cannot overcome the hemodynamic effects of ongoing OC-induced RAS activation. Augmented tissue mRNA levels of eNOS supports our assertion that the NO system is activated in the OC users.

OC use increases basal production and release of NO in the brachial artery (5) in some studies (21). For example, John and coworkers (21) studied changes in forearm blood flow in OC users and nonusers, and our second major finding was that L-arginine infusion reduced circulating ANG II levels in both groups of subjects. De Nicola and coworkers (10) have demonstrated important ANG II-NO glomerular interactions in rats, suggesting that NO synthesis is activated by, and then functions as a physiological antagonist of, ANG II (10, 17, 39). ANG II-NO interactions have been suggested by others in in vitro and in vivo experiments (49, 51), and NO may abolish ANG II-dependent vascular and mesangial contraction through the intracellular actions of cGMP (49). Our findings are consistent with a NO-mediated negative feedback loop on ANG II, since ANG II levels decreased in a stepwise fashion in response to a graded infusion of L-arginine and then returned toward normal when the L-arginine was discontinued. Although the mechanism(s) responsible for this feedback loop was not elucidated in the present study, it may be hemodynamically mediated (10, 17, 39, 49, 51).

The OC users also displayed elevated baseline aldosterone levels, without evidence of systemic hypertension or renal vasoconstriction. NO activation is thought to be an important factor that protects against the development of hypertension during states of chronic mineralocorticoid excess (14, 43), such as during OC use (36–38, 55). It does so through both hemodynamic and diuretic mechanisms in dogs (14), thereby blunting the long-term hypertensive effects of mineralocorticoids in most settings. The blunted effect of L-arginine infusion in the OC group supports the concept that the NO system is upregulated and serves to modulate hemodynamic function in the setting of high-circulating aldosterone and ANG II levels.

Our last major observation was that tissue eNOS expression was augmented in OC users. It is surprising that, despite this
future study.

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