Effects of Two Oral Contraceptives on Plasma Levels of Nitric Oxide, Homocysteine, and Lipid Metabolism

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This prospective study was performed to investigate the effect of 2 low-dose oral contraceptives containing a second- and a third-generation progestagen on factors, which may influence the individual cardiovascular risk by regulating endothelial function and development of atherosclerosis. Sixteen women were randomized to receive 3 cycles of treatment with 30 μg ethinylestradiol/150 μg levonorgestrel (EE/LNG) and 3 cycles of treatment with 30 μg ethinylestradiol/75 μg gestodene (EE/GSD). Before and after treatment the plasma levels of nitric oxide (NO), homocysteine, cholesterol, high-density lipoprotein (HDL), and triglycerides were measured. No significant alterations of the NO, homocysteine, and triglyceride plasma levels were observed during use of both contraceptive pills. Compared to levels after EE/LNG treatment, HDL plasma levels were higher ($P = .05$) and the cholesterol/HDL ratio was lower after the EE/GSD pill ($P = .05$). Significant correlations were found between NO and homocysteine and NO and cholesterol. Our data indicate that the cardiovascular risk associated with these contraceptive pills may not be explained by a negative influence on NO or homocysteine.

HOMOCYSTEINE, a sulfur-containing amino acid, is an intermediate metabolite of methionine. An elevated homocysteine plasma concentration is an independent risk factor for cardiovascular disease.[1-4] Hyperhomocysteinemia is common in patients with peripheral arterial disease, cerebrovascular disease, and venous thromboembolism.[2,5-7] The role of homocysteine in the pathogenesis of endothelial dysfunction is not completely understood. One of the mechanisms discussed is a decreased availability of the vasodilator nitric oxide (NO) in subjects with high homocysteine levels,[8] a hypothesis which is supported by in vitro investigations[9] and experimental findings in animals and humans[10,11] demonstrating an association between elevated homocysteine levels and increased concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase. NO is synthesized in the vascular endothelium from L-arginine. It induces vasodilatation and inhibits platelet aggregation and smooth muscle cell aggregation and migration.[12] Decreased bioavailability of NO may result in abnormal reactions between the vessel wall and platelets and is thus involved in the initiation and progression of atherosclerosis.[12,13] There is evidence for endothelial dysfunction and specific defects in NO-mediated vasodilatation in patients with hypercholesterolemia or hypertension, smokers, and individuals with raised levels of homocysteine.[14-17] Several studies have indicated that female sex steroids may affect NO and homocysteine.[18-24]

The use of combined low-dose oral contraceptives (COCs) is associated with an increased risk of myocardial infarction, especially in women with cardiovascular risk factors like smoking or hypertension.[25-30] Some studies indicate that the incidence of myocardial infarction is substantially higher in users of second-generation compared to third-generation COCs,[31] while others failed to demonstrate a different risk.[30] COCs contain the estrogen 17-α-ethinylestradiol, which has been shown to upregulate the transcription of endothelial nitric oxide synthase (eNOS) in human endothelial cells.[32] The aim of the present prospective study was to investigate the influence of a long-term treatment with 2 COCs containing different progestagens on plasma levels of NO and homocysteine. Because lipoproteins are known to alter the concentration of homocysteine and NO,[33-37] we also measured plasma lipids.

SUBJECTS AND METHODS

Participants in this study were 16 healthy premenopausal women (mean age, 21.2 years; range, 18 to 30) with regular menstrual cycles recruited from our family planning clinic. All women wished to use COC pills for birth control. Exclusion criteria were hormonal treatment during the last 3 months, smoking, treatment with vitamin preparations, birth or lactation less than 6 months previously, family history of cardiovascular disease, contraindications against the use of oral contraceptives, alcohol abuse, and the need for regular medication. Before inclusion, participants underwent a gynecologic examination with PAP smear. Only women with a normal gynecologic status were included. Informed consent was obtained from all subjects and the study was approved by the hospital ethics committee.

Blood pressure was checked before starting the contraceptive pills and after 3 treatment cycles. Subjects were randomized to receive either 30 μg ethinylestradiol/150 μg levonorgestrel (EE/LNG) (group A) or 30 μg ethinylestradiol/75 μg gestodene (EE/GSD) (group B). After 3 treatment cycles, the patients were crossed over until the end of month 6. Each group included 8 participants.

All blood samples were taken in the morning after a fasting period of 12 hours and after a diet of 24 hours. The diet excluded the use of the following foodstuffs, which are known to influence the plasma levels of NO or homocysteine: cheese, meat, salad, spinach, cabbage, canned food, tea, and alcoholic drinks.

Plasma samples for the measurements of homocysteine, NO, cholesterol, high density lipoprotein (HDL), triglycerides, estradiol, and progesterone were taken as follows: sample 1 during the follicular phase (days 10 to 12) and sample 2 during the luteal phase (days 20 to 23) of the baseline cycle, sample 3 after 3 pill cycles (pill-day 19 to 21), sample 4 in the pill-free interval after 3 pill cycles (pill-free day 6 or 7), and sample 5 at the end of the 6th treatment cycle (pill-day 19 to 21). Plasma was separated within 30 minutes and then stored at -20°C until assayed.

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Assays

Nitrate/nitrite as stable metabolites of NO were measured by a fluorometric assay (Cayman Chemical Co, Ann Arbor, MI) (intra-assay coefficinet of variation [CV], 2.2%; interassay CV, 2%; detection limit, 7.8 μmol/L). Plasma total homocysteine concentrations were determined using high-performance liquid chromatography with fluorescence detection (intra-assay CV, 4%; interassay CV, 4.2%; lower limit of detection, 1 μmol/l). Total homocysteine includes the sum of homocysteine, cysteine-homocysteine mixed disulfide, and the cysteinyl moieties of homocysteine. Estradiol and progesterone were analyzed by commercially available radioimmunoassays (Sorin Biomedica Diagnostics, Saluggia, Italy: intra-assay CV, 4.2%; interassay CV, 4.9%; sensitivity, 18 pmol/L; and Diagnostic Products Corp, Los Angeles, CA: intra-assay CV, 3.6%; interassay CV, 3.9%; sensitivity, 0.06 nmol/L). Cholesterol, triglycerides, and HDL were measured automatically (Hitachi 747; Roche Diagnostica, Basel, Switzerland).

Statistical Analysis

Data are given as means ± SD. Baseline parameters for both groups were compared by unpaired *t* test. Baseline and follow-up measurements within each group were compared by Wilcoxon signed-rank test, because the data were not normally distributed. To evaluate the effect of a COC containing 30 μg EE on the investigated parameters we included all women. The separate analyses of group A and B allowed us to determine whether the 2 different progestagens in these pills might exert a different effect on the measured markers. Simple and multiple linear regression analyses were used to determine the correlation between the plasma levels of NO, homocysteine, estradiol, progesterone, and plasma lipids. These calculations were done for all samples and separately for the samples taken in treatment-free intervals (samples 1, 2, 4). To evaluate whether the changes of the investigated parameters were correlated, we calculated the difference of each value from the mean cycle value [(sample 1 + sample 2)/2]. A P value of .05 was considered statistically significant. All analyses were performed using Statview 4.01 data analyses software (Abacus Concepts, Berkeley, CA).

RESULTS

Baseline characteristics of the subjects are listed in Table 1. Fifteen participants completed the study and 1 woman (group B) stopped the medication after 3 treatment cycles. There were no differences in the baseline parameters between the participants of both groups (Table 1).

Table 1. Baseline Characteristics of 16 Healthy Premenopausal Women Before Treatment With Two Different Oral Contraceptives

Variable	All	Group A	Group B
Treatment cycle 1-3		EE/LNG	EE/GSD*
Treatment cycle 4-6		EE/GSD	EE/LNG
No. of women	16	8	8
Age (yr)	21.2 (3.8)	20.3 (3.7)	22.0 (4.0)
Blood pressure			
Systolic (mm Hg)	116 (12)	117 (14)	116 (11)
Diastolic (mm Hg)	64 (6)	62 (6)	66 (7)
Body mass index (kg/m^2)	22.6 (2.8)	23.0 (3.2)	22.0 (2.3)
Estradiol level (pg/mL)			
Cycle day 10-12	212 (192)	112 (41)	291 (228)
Cycle day 20-23	401 (222)	421 (213)	383 (243)
Progesterone level			
Cycle day 10-12	3.7 (4.4)	2.2 (0.6)	4.8 (5.8)
Cycle day 20-23	21.3 (23.9)	11.3 (12.9)	30.1 (28.5)

NOTE. Values are means (SD).

Abbreviations: EE/LNG, 30 μg ethinylestradiol (EE)/150 μg levonorgestrel; EE/GSD, 30 μg EE/75 μg gestodene.

Treatment with the 2 contraceptive pills did not cause an increase of blood pressure. Table 2 shows the plasma levels of NO, homocysteine, cholesterol, HDL, and triglycerides and the cholesterol/HDL ratio during the baseline cycle and during treatment with both contraceptive pills. There were no significant changes in NO plasma levels after 3 and 6 pill cycles compared to the baseline cycle (Fig 1). The analyses of the subgroups revealed no significant effect of either COC on NO. Homocysteine plasma levels were not different during the follicular and luteal phase of the menstrual cycle or after treatment with both COCs (Fig 1). In each subgroup, homocysteine plasma levels were significantly lower after 3 cycles of EE/GSD compared to the levels during the follicular phase of the baseline cycle (group A, $P < .05$; group B, $P < .05$), but not compared to the levels determined during the luteal phase (Table 2). Triglycerides and cholesterol did not change significantly during the study (Table 2). In the separate analyses of group A and B, HDL was significantly higher and the cholesterol/HDL ratio was significantly lower ($P < .03$) after treatment with EE/GSD compared to EE/LNG. All correlation analyses were made for the samples taken in treatment-free intervals (samples 1, 2, 4) and for all samples (samples 1 through 5). There was a significant correlation between NO and homocysteine in the samples without and during hormonal treatment ($P < .005$, $r = 0.41$ and $P < .007$, $r = 0.39$, respectively) (Fig 2). Additionally, there was a significant correlation between NO and cholesterol during the baseline cycle ($P < .03$, $r = 0.36$) and during hormonal treatment ($P < .03$; $r = .27$) (Fig 2). Furthermore, the association between NO and the cholesterol/HDL ratio was significant ($P < .03$, $r = 0.37$ before treatment; $P < .05$; $r = 0.24$ during treatment). No correlation was found between NO or homocysteine and the parameters estradiol, progesterone, HDL, and triglycerides.

DISCUSSION

The current work was designed to elucidate the effect of 2 COCs on the plasma levels of homocysteine and NO. Both parameters are known to be influenced by sex hormones and play a key role in the development of atherosclerosis.[5-7,12,13] In vitro and in vivo data indicate that the plasma levels of NO and homocysteine are partially controlled by cholesterol and HDL,[10,11] and the levels of both lipoproteins are known to change during oral contraceptive use.[38-40]

In the present study we found that long-term use of both pill preparations did not cause significant alterations in the plasma levels of NO and homocysteine. In the analyses of the subgroups, homocysteine levels after EE/GSD treatment were lower than during the follicular but not the luteal phase of the baseline cycle. However, because the homocysteine levels during the follicular phase and the luteal phase were not different, and because we did not find significant differences in homocysteine levels after EE/LNG or EE/GSD in each subgroup, it cannot be concluded that the 2 COCs exerted a different effect on homocysteine, nor that EE/GSD causes a decrease of homocysteine levels.

Table 2. Plasma Levels of NO, Homocysteine, and Lipoproteins for 16 Premenopausal Women Before and During Treatment With Two COCs

Variable	Control Cycle		Treatment Cycle		
	Cycle Days 10-12	Cycle Days 20-23	Cycle 3: Days 18-21	Cycle 3: Days 26-28	Cycle 6: Days 18-21
NO levels (μmol/L)					
All (N = 16)	61.3 (32.1)	57.7 (33.7)	61.4 (38.1)	55.6 (30.9)	54.7 (38.8)
Group A (n = 8)	67.2 (26.7)	69.6 (39.5)	70.4 (38.3)	63.1 (28.6)	63.0 (46.9)
Group B (n = 8)	56.6 (36.7)	47.3 (26.0)	54.4 (38.7)	49.7 (33.1)	47.7 (32.5)
Homocysteine (μmol/L)					
All (N = 16)	10.67 (5.0)	9.75 (3.4)	9.66 (4.4)	10.12 (4.1)	11.06 (4.5)
Group A (n = 8)	9.89 (2.6)	9.64 (2.5)	9.46 (2.9)	9.15 (3.0)	9.70 (3.2)*
Group B (n = 8)	11.28 (6.4)	9.85 (4.2)	9.85 (5.6)*	10.9 (4.8)	12.43 (5.4)
Cholesterol (mmol/L)					
All (N = 16)	4.72 (0.7)	4.60 (0.9)	4.69 (1.0)	5.29 (0.9)	4.81 (0.9)
Group A (n = 8)	4.59 (0.6)	4.61 (0.9)	4.59 (0.7)	4.91 (0.6)	4.81 (0.7)
Group B (n = 8)	4.81 (0.8)	4.58 (1.0)	4.77 (1.3)	5.74 (1.0)	4.80 (1.1)
HDL-cholesterol (mmol/L)					
All (N = 16)	1.48 (0.3)	1.50 (0.6)	1.49 (0.3)	1.49 (0.3)	1.47 (0.4)
Group A (n = 8)	1.50 (0.4)	1.65 (0.6)	1.45 (0.3)	1.54 (0.3)	1.79 (0.4)*†
Group B (n = 8)	1.47 (0.3)	1.32 (0.5)	1.51 (0.4)*	1.44 (0.3)	1.19 (0.2)
Cholesterol/HDL ratio					
All (N = 16)	3.43 (0.8)	3.21 (0.8)	3.35 (0.8)	3.70 (0.9)	3.73 (1.4)
Group A (n = 8)	3.21 (0.7)	2.86 (0.6)	3.25 (0.7)	3.35 (0.7)	2.47 (0.6)*
Group B (n = 8)	3.56 (0.9)	3.64 (0.8)	3.42 (0.8)*	4.12 (1.0)	4.57 (1.3)
Triglycerides (mmol/L)					
All (N = 16)	0.94 (0.3)	0.86 (0.2)	1.10 (0.5)	0.97 (0.4)	1.04 (0.3)
Group A (n = 8)	0.99 (0.3)	0.85 (0.2)	0.87 (0.1)	0.81 (0.2)	1.03 (0.1)
Group B (n = 8)	0.89 (0.3)	0.86 (0.3)	1.26 (0.7)	1.17 (0.4)	1.04 (0.3)

NOTE. Group A, cycles 1 to 3: 30 μg ethinylestradiol/150 μg levonorgestrel (EE/LNG); cycles 4 to 6: 30 μg ethinylestradiol/75 μg gestodene; Group B, cycles 1 to 3: 30 μg ethinylestradiol/75 μg gestodene; cycles 4 to 6: 30 μg ethinylestradiol/150 μg levonorgestrel. Data are presented as means (SD).

*$P \leq .05$ *v* 3 cycles of treatment with EE/LNG.

† $P \leq .05$ *v* control cycle days 10 to 12.

17-α-Estradiol increases endothelial NO production in cultured cells[41] and reduces inducible nitric oxide synthase in smooth muscle cells of rat aorta.[42] Both of these mechanisms may attribute to the prevention of atherosclerosis.[43] In postmenopausal women, estrogen-replacement therapy causes an increase in NO plasma levels,[22,23] an effect that is attenuated by the addition of progestagens.[22] The influence of 17-α-ethinylestradiol and COCs on NO is less well studied. 17-α-Ethinylestradiol enhances the expression of eNOS mRNA in human endothelial cells[32]; therefore, it could be expected that treatment with the estrogen ethinylestradiol, which has a high affinity to the estrogen receptor, might increase the plasma levels of NO. However, no such changes were observed during long-term treatment with a combination of ethinylestradiol with a progestagen in the present study. It could be that the progestagen component of the pill counteracts a positive effect of ethinylestradiol on NO. Because a previous study demonstrated that short-term treatment with ethinylestradiol 50 μg alone does not cause a significant increase of NO,[44] this explanation is rather unlikely. An alternative hypothesis is that estrogen treatment of healthy premenopausal women with sufficiently high estrogen and NO levels does not cause a further rise in the NO plasma concentration. From the physiologic point of view, this explanation makes sense, because unphysiologically high NO levels may cause endothelial injury instead of protection.[45]

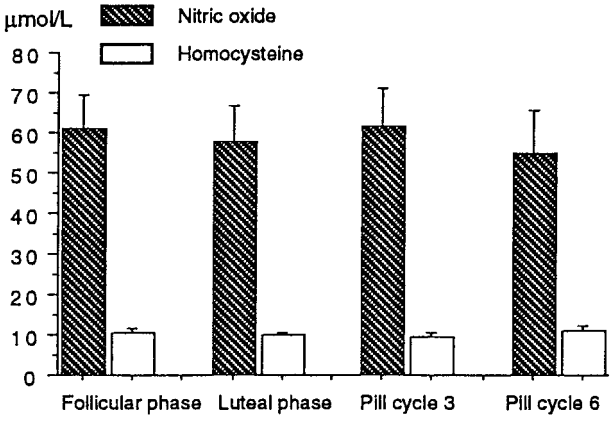

Fig 1. NO and homocysteine plasma levels during the follicular and luteal phases of the menstrual cycle and after 3 and 6 cycles of treatment with 2 COCs containing 30 μg ethinylestradiol/150 μg levonor-gestrel or 30 μg ethinylestradiol/75 μg gestodene. Values are means ± SD.

This is the first prospective study to investigate the effect of COCs on homocysteine. Previous case-control studies presented conflicting results. While most observed no effect of COCs on plasma homocysteine,[46,47] an increase of homocysteine only in the pill-free interval was demonstrated in one small case-control study.[48] In postmenopausal women, the de-

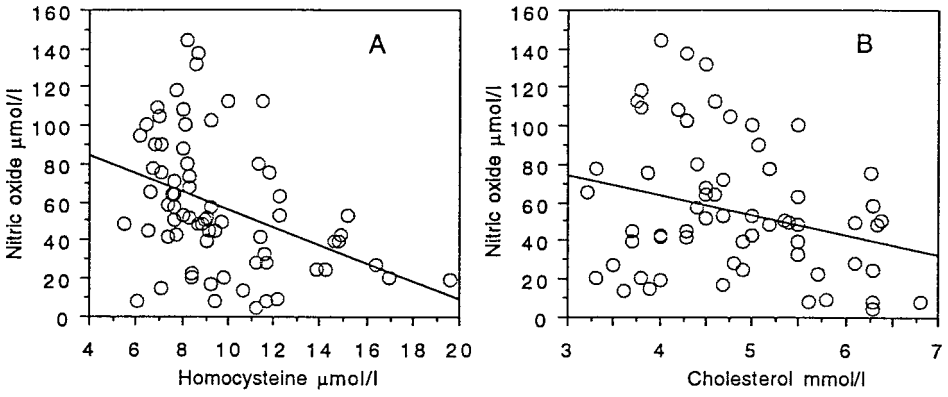

Fig 2. Regression analyses between (A) NO and homocysteine (r = 0.39, P = .0007), and (B) NO and cholesterol (r =0.27, P = .03) in healthy women. Data include those of the normal cycle and during treatment with 2 COCs.

crease in homocysteine following estrogen-replacement therapy is approximately 10%.[20,21] A possible reason for the failure of homocysteine levels to decrease in our patients could be the different hormonal status of premenopausal and postmenopausal women. Estrogens and possibly synthetic progestagens might decrease homocysteine in estrogen-deficient women, but according to our results do not cause a further decrease of physiologic homocysteine levels in premenopausal women. This interpretation is in accordance with previous findings demonstrating that homocysteine levels do not change during the menstrual cycle and during short-term treatment with ethinylestradiol.[44]

Hyperhomocysteinemia is associated with impaired endothelial function in animals[49] and humans.[50,51] The mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia remain unclear,[52] although there is some evidence for an interaction between homocysteine and NO. In high concentrations, homocysteine may decrease endothelial production of NO through oxidative mechanisms[53,54] and is directly toxic to cultured endothelial cells.[55] Böger et al[10] found elevated plasma concentrations of ADMA, an endogenous inhibitor of nitric oxide synthase, in monkeys with hyperhomocysteinemia. The observation of a highly significant correlation between NO and homocysteine in our study provides further evidence for an interaction of these parameters in vivo. Treatment with the oral contraceptives EE/LNG or EE/GSD did not have a negative impact on this association, which may indicate that the balance between NO and homocysteine is not disturbed by oral contraceptive treatment.

Hypercholesterolemia-induced vascular disease and atherosclerosis are characterized by an early selective decrease in the bioavailability of endothelium-derived NO.[56] Increased production of superoxide in hyperlipidemia[57] causes an increased inactivation of NO by radicals,[58] which is the reason for the decrease in NO plasma levels associated with rising cholesterol levels. Therefore, the negative association between NO and cholesterol observed in our study is in accordance with the present knowledge on the mechanisms regulating the plasma levels of these 2 parameters.

COC preparations containing levonorgestrel in a dosage as it was used in the present study cause a small decrease in HDL and a nonsignificant increase in low-density lipoprotein (LDL),[40] while EE/GSD has been shown to induce a significant increase in HDL and apolipoprotein A-I (apoA-I) without a concomitant alteration in LDL.[38,39] In the present study, HDL levels increased during EE/GSD treatment, but this was only significant in group A. HDL reduces atherosclerosis in animal models and in association with the overexpression of apoA-I.[60,61] It has been demonstrated to be a strong inverse predictor for atherosclerosis.[62] Recent evidence suggests that HDL can inhibit the formation of oxidized LDL[63,64] and decrease the extent of post-translational protein homocysteinylation,[65] both mechanisms protecting against vascular damage. However, the higher HDL levels and the lower HDL/cholesterol ratio observed in our study during use of the gestodene preparation were not associated with concomitant measurable changes in NO or homocysteine. The degree to which the observed changes in plasma lipids during EE/GSD treatment alone cause a vasoprotective effect can only be speculated.

In conclusion, long-term treatment with 2 COCs did not have a negative impact on NO or homocysteine plasma levels. Our results do not support the hypothesis that the increased risk for cardiovascular diseases associated with contraceptive pills can be attributed to changes in plasma levels of NO and homocysteine.

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