Hormonal Contraceptive Increases Plasma Lipid Peroxides in Female Rats
Relationship to Platelet Aggregation and Lipid Biosynthesis

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We investigated whether changes in plasma oxidative properties could occur after oral (hormonal) contraceptive (OC) administration in female rats and whether such changes could be responsible for the platelet increase in aggregation and lipid biosynthesis observed with that treatment. Platelets and plasma (platelet-poor) from control and OC (ethinyl estradiol + lynestrenol)-treated rats were prepared separately. Thrombin-induced aggregation of control platelets was markedly enhanced after incubation for 4 (p<0.025) to 60 (p<0.001) minutes in OC as compared with control plasma. Under the same conditions, platelet lipid biosynthesis was increased also (p<0.05 to p<0.01), but after 3 hours incubation. The enhanced response of platelets to aggregation induced by OC plasma could be inhibited by adding either glutathione (p<0.025), vitamin E (p<0.025), catalase (p<0.05), or peroxidase + glutathione (p<0.005) to plasma or 2,6-di-bis(ter-butyl)p-cresol (p<0.05) to platelets before incubation. The peroxidized free fatty acids isolated from OC plasma added to normal platelets induced a 150% (p<0.001) increase in the response to thrombin as compared with the fatty acids from control plasma. In addition, the level of malondialdehyde and conjugated dienes was significantly (p<0.02 to p<0.001) increased in OC compared with control plasma. We conclude that the enhanced formation in plasma of lipid hydroperoxides seems to be the initial event stimulating platelets after OC treatment, at least in rats. (Arteriosclerosis 9:84—89, January/February 1989)

Probably in relation to their known predisposing effect to thrombosis, oral (hormonal) contraceptives (OC) induce functional and metabolic abnormalities in platelets, as reported in women1,2 and rats.3 After OC administration, we have observed a close relationship between the response of platelets to aggregation and the consequent increase in platelet lipid biosynthesis, mostly the lanosterol + dihydrolanosterol fractions,4,5 precursors of cholesterol. These fractions have been associated with platelet hyperaggregability, especially to adenosine 5'-diphosphate (ADP).6

Moreover, the enhanced aggregability and lipid biosynthesis were observed only on polyunsaturated fat-rich diets7,8 and were inhibited by vitamin E administered with the estrogen in rat7 and the contraceptive in women.9 Plasma vitamin E was low in estrogen-treated rats,6 as in women using OC.8,9,10 Because vitamin E is known to antagonize lipid peroxidation in plasma,11 OC could induce oxidative phenomena, eventually playing a role in the platelet abnormalities mentioned above. The purpose of the present study was to explore whether changes in plasma oxidative properties could occur after OC administration, and whether such changes could be involved in the platelet increase in aggregation and lipid biosynthesis observed with that treatment.

Methods

Chemicals

U14C acetic acid sodium salt was obtained from the Commissariat à l'Energie Atomique (Saclay, France); ethinyl estradiol and lynestrenol were from the Biochemical Division of ICN (Cleveland, OH); peroxidase (horse-radish) and catalase were from Boehringer-Mannheim Company, West Germany; reduced glutathione was from the Sigma Company (St. Louis, MO); and 2,6-di-bis(ter-butyl)p-cresol (BHT) was from Fluka Company. Trolox (soluble vitamin E) was a gift from Hoffman-La Roche (Basel, Switzerland).

Animals and Treatment

For each experiment we used six female Sprague-Dawley rats weighing 300 to 350 g fed laboratory chow. The animals in the control group received once on the first day, by stomach tube, 0.5 ml of olive oil per 100 g body weight. The OC group received 0.5 ml olive oil containing 30 µg ethinyl estradiol and 750 µg lynestrenol as determined in previous studies.12 That dosage of OC is three-eighths the dosage used routinely to block ovulation in adult female rats (P. J. Van der Vies, Organon Company, Oss, The Netherlands, personal communication). Blood was taken from the jugular vein of fasted animals under ether anesthesia on the fourth day after beginning treatment.

Platelet Preparation

Blood was collected with the following anticoagulant: 0.8 g citric acid; 2.45 g glucose, 2.2 g sodium citrate, and

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Received February 8, 1988; revision accepted August 1, 1988.
Distilled water to 100 ml (0.5 ml of anticoagulant for 1.5 ml of blood). Platelets were separated from red and white cells by centrifugation as previously described. Then 4 x 10^9 platelets were incubated in different plasma preparations (2 ml) as indicated below. After the incubations, platelet-rich plasma (PRP) was centrifuged (1200 g) for 15 minutes. The platelets were gently washed twice and resuspended in Tyrode's solution containing 8.7 g sodium chloride, 0.2 g potassium chloride, 0.8 g sodium bicarbonate, 1 g glucose, 0.07 g sodium dihydrogen orthophosphate, 1.2 g magnesium chloride, 2.5 g gelatin, and distilled water to 1000 ml adjusted to pH 7.4.

In a series of experiments, platelets, before incubation in plasma, were treated for 15 minutes with BHT as follows: To 1 ml of PRP was added 6 µl of BHT (0.1 M) in absolute ethanol; ethanol alone was added to the control PRP. Platelets were then centrifuged and the pellet treated as above.

**Plasma Preparation**

From each animal, blood was drawn into three plastic (BD plastic) 2-ml syringes containing 0.2 ml of 3.8% sodium citrate as the anticoagulant. Platelet-poor plasma (PPP) was prepared by sequential centrifugations:

1. PRP was obtained after spinning blood (150 g) for 10 minutes at 4°C.
2. To obtain PPP, PRP was centrifuged for 20 minutes (1200 g) to remove most platelets without damage. An additional centrifugation was performed (2000 g, 10 minutes) to remove the few contaminant cells. PPP was prepared from the control group and the OC animals for immediate use or stored (~30°C) and used within a month.

The PPP (2 ml, unless otherwise indicated) was tested on platelet function, either as obtained or after pretreatment for 2 minutes with the following additives: 1) reduced glutathione (2 mM final concentration), 2) soluble vitamin E (1.5 mM final), 3) horseradish peroxidase (1.4 U/0.5 ml) + reduced glutathione (2 mM final) (in some experiments, glutathione peroxidase was used instead of horseradish peroxidase, with similar results), and 4) catalase (1.5 U/0.5 ml). Control platelets (2 x 10^9/ml) were incubated at 25°C for 4, 10, 30, and 60 minutes (untreated plasma) or for 4 minutes (pretreated plasma). Incubated platelets were washed and resuspended in Tyrode's solution and finally tested for aggregation as described below.

**Platelet Aggregation**

Platelet aggregation was performed on platelets (500 000/µl) resuspended in a Tyrode's solution (pH 7.4) containing gelatin. The aggregating agents used were thrombin (from human plasma, Sigma Chemical Company), final concentration 0.12 U/ml, and ADP sodium salt (Sigma Chemical Company), final concentration 0.8 x 10^-5 M.

**Radioactive Incubations**

Platelets (2 x 10^9) from control and OC-treated female rats were incubated at 37°C for 3 hours (1 hour in three preliminary experiments) in 2 ml of PPP from either control or OC-treated animals. Twenty µl of an aqueous solution of 14C-acetate (10 µCi) was added to the plasma. After incubation, platelets were washed twice in Tyrode's solution, as indicated above; lipids were extracted and separated by thin-layer chromatography, as reported elsewhere. Radioactivity was then evaluated in each of the fractions.

**Plasma Lipids**

Plasma free fatty acid peroxidases were isolated from control and OC plasma. First, free fatty acids were extracted by the technique of Antonis and dissolved in petroleum ether. Peroxidases were further extracted by ethanol (70%) from the petroleum ether extract as recommended by Gray and Barrowcliffe. The ethanol fraction obtained, kept on ice, was tested immediately for platelet functions as follows: 4 µl of the ethanol fraction containing peroxidase was added to 500 µl of a platelet suspension (500 000/µl) 1 minute before inducing aggregation. Total lipids were determined in control and OC plasma by an enzymatic technique (Kit, Biomerieux, France). The free fatty acids were measured by an enzymatic and colorimetric technique (Nafa C-test Wako, Wako Pure Chemical Incorporated, Ltd., Osaka, Japan).

Malondialdehyde (MDA) and conjugated dienes were evaluated in plasma obtained from blood taken with 3.8% sodium citrate (pH 7.4) (0.2 ml for 1.8 ml of blood) as the anticoagulant and centrifuged without delay for 15 minutes at 2500 g. MDA was determined immediately after sample preparation in 0.5 ml of plasma with the thiobarbituric technique in the presence of BHT as described by Quintanilha and Packer. Conjugated dienes in lipids were also measured at once by ultraviolet (233 nm) absorption, as indicated by the same authors.

**Statistical Analysis**

Student's t test, paired or unpaired, was used to evaluate the significance of the data obtained.

**Results**

**Influence of OC Platelet-Poor Plasma on Aggregation of Control Platelets**

Results are given in Figure 1. After 4, 10, 30, and 60 minutes of incubation in OC plasma, control platelets exhibited a significant (p < 0.05 to p < 0.001) increase to thrombin-induced aggregation. After 4, 10, or 30 minutes of incubation, the response of platelets to thrombin was increased by 86% to 108% and by 21% after 60 minutes. The ADP-induced aggregation was also slightly enhanced, but the difference from the controls was not statistically significant.

As shown in Figure 2, the treatment of OC plasma by reduced glutathione, soluble vitamin E, peroxidase + reduced glutathione, or catalase inhibited markedly the stimulating effect of OC plasma on the thrombin-induced aggregation of control platelets. The most significant inhibitory effect was induced by glutathione and the combined treatment peroxidase + glutathione, which completely blocked the effect of the OC plasma.

When the control platelets were treated with BHT before incubation with OC plasma, the enhancing effect of
Radioactive Incubations

Results obtained are reported in Table 1. Platelets were incubated for 3 hours in plasma in four different ways: 1) control platelets in control plasma, 2) OC platelets in OC plasma, 3) control platelets in OC plasma, and 4) OC platelets in control plasma. A significant increase of °C-aceate incorporation into platelet total lipids was observed in incubation 2 (plasma and platelets from OC) as compared with incubation 1 (control plasma and platelets). After radioactive platelet lipid separation by thin-layer chromatography, seven radioactive spots were identified: phospholipids, monoglycerides, cholesterol, diglycerides, lanosterol + dihydrolanosterol, free fatty acids, and triglycerides + esterified cholesterol. An increased radioactivity was noted in all lipid fractions after treatment by OC. However, only cholesterol, diglycerides, and lanosterol + dihydrolanosterol were significantly increased, these last fractions by about tenfold. The acetate incorporation was also increased in incubation 3, but concerning the lanosterol fraction, the results were not significant at the same extent, since only a twofold increase in the incorporation was noted. In incubation 3, all the lipid fractions presented an increased radioactivity. However, only the increases in phospholipids, total lipids, and lanosterol were statistically significant. By contrast, when OC platelets were incubated in control plasma (incubation 4), the OC platelets were not significantly modified, except for the °C-aceate incorporation into phospholipids when compared with OC platelets incubated in their own plasma. Otherwise, the radioactivity remained elevated in those platelets, especially in the lanosterol and dihydro-

Lanosterol fractions. When the incubation period was only 1 hour, we did not observe any significant change in radioactive lipids of incubation 3 as compared with incubation 1 (data not shown).

Plasma Lipids

The influence of free fatty acid peroxidases from control and OC plasma was evaluated on thrombin and ADP-induced aggregation of control platelets. As shown in Table 2, the free fatty acid peroxidases isolated from OC plasma increased the response of platelets to thrombin highly significantly and to ADP aggregation less significantly. In Table 3 are reported the levels of various plasma lipid fractions. Although total lipids were markedly decreased after OC treatment, free fatty acids were unaffected. By contrast, MDA and, especially, conjugated
The present study demonstrates that the hyperaggregability to thrombin of platelets from female rats treated with OC is induced by plasma changes that are able to modify normal platelets within minutes. However, the ensuing platelet modifications appear to be permanent since they can still be observed once the platelets are resuspended in Tyrode's solution. Our results also suggest that the mechanism involved is an oxidative process, since addition to plasma or platelets of substances able to prevent peroxidation or its damage to cells also prevents the hyperaggregability.

Catalase and vitamin E added to OC plasma were able to decrease significantly the ensuing response of platelets to aggregation, but some effect of the OC plasma could still be observed. By contrast, glutathione (with or without peroxidase) was able to completely block the hyperaggregability induced by the OC plasma.

Vitamin E is known to lower lipid peroxides, catalase to destroy specifically H$_2$O$_2$. Glutathione-peroxidase + reduced glutathione is the only enzymatic system that reacts with both H$_2$O$_2$ and lipid hydroperoxides. As a result, this combined treatment was the most efficient to prevent the potentiating effect of OC plasma on platelets. Nevertheless, glutathione alone was already quite effective in that connection. Glutathione is known to protect membranes from oxidative damage independently of peroxidase activity, apparently by preventing peroxidation rather than by reducing the lipid hydroperoxides already formed. Alternatively, glutathione could use an endogenous plasmatic peroxidase recently characterized.

Under our experimental conditions BHT was also markedly effective to protect platelets from the damage induced by OC plasma. BHT is also known to impair peroxide-induced lesions in erythrocyte membranes. Whatever the mechanisms involved in the protective effects of vitamin E, catalase, glutathione, and BHT, it seems clear that they all impede either lipid peroxidation or oxidative damage to membranes.

The presence of lipid hydroperoxides has already been reported in human plasma, and even slight elevations of this material can be expected to have undesirable effects in biological processes. Under our experimental conditions, we have observed that the free fatty acid peroxides from rat OC plasma were able to induce a threefold increase in the response of platelets to thrombin. The significantly higher level of MDA in the OC plasma strongly suggests that lipid peroxidation occurred in these plasma. However, because the thiobarbituric reaction to determine MDA is very sensitive but has a low specificity, those results had to be confirmed by the evaluation of the conjugated dienes, formed in direct proportion to the level of lipid hydroperoxides. As a matter of fact, when referring to total lipids, which are drastically reduced in OC-treated rats, we noted a twofold increase in conjugated dienes in the OC plasma. This result explains why antioxidants such as vitamin E can prevent platelet hyperactivity when added in vitro to plasma, as in the present study or when administered to rats or to women receiving hormonal contraceptives.

In previous work, we have shown in rats as well as in women that OC can induce hyperaggregability mostly when the diet is rich in polyunsaturated fat. The ensuing result is an increase in the level of platelet cholesterol and of lanosterol (plus dihydrolanosterol) biosynthesis. Lanosterol added to platelets increases their response to thrombin and ADP-induced aggregation. Cholesterol has been shown, when drastically enhanced in platelets, to increase the response only to ADP. Nevertheless, the problem is by what mechanism does the estrogen, since it is this component that in hormonal contraceptive induces the platelet hyperactivity, stimulate cholesterol accumulation and lipid biosynthesis. We have observed that it is not a direct effect of the ethinyl estradiol on platelets and that it takes several days to be observed after its administration. It is also blocked by vitamin E. The present study shows that within a few minutes normal platelets incubated in plasma from animals treated with OC become hyperresponsive to thrombin-induced aggregation as platelets from OC-treated animals. The fact that prostaglandin biosynthesis can be modulated by lipid hydroperoxides considered as the major activator material.
Table 1. Plasma Influence of Oral Contraceptive-treated Female Rats on Acetate Incorporation Into Platelet Lipids

| Lipid                        | 1           | 2           | 3           | 4           |
|------------------------------|-------------|-------------|-------------|-------------|
| Total lipids                 | 24.810 ± 5.259 | 40.923 ± 15.332 | 31.255 ± 8.457 | 35.142 ± 9.757 |
| Phospholipids                | 12.963 ± 3.254 | 16.284 ± 6.797 | 16.233 ± 4.198  | 12.921 ± 5.690 |
| Monoglycerides               | 3.676 ± 9.17 | 5.005 ± 15.18 | 4.139 ± 16.48 | 4.686 ± 11.75 |
| Cholesterol                  | 3.10 ± 10.8 | 8.25 ± 48.3  | 4.13 ± 16.7   | 9.24 ± 652  |
| Diglycerides                 | 4.37 ± 31.8 | 8.34 ± 309.9 | 5.00 ± 273   | 9.68 ± 553  |
| Lanosterol +                 | 9.07 ± 5.55 | 9.964 ± 6338 | 19.71 ± 1009 | 6.079 ± 4392 |
| Free fatty acids             | 12.38 ± 17.42 | 22.25 ± 1666 | 26.28 ± 2439 | 18.35 ± 1239 |
| Triglycerides +              | 4.33 ± 121.6 | 56.22 ± 2910 | 5.35 ± 1821 | 549.9 ± 1608 |
| cholesterol ester            |             |             |             |             |

Values are cpm/2 x 10^6 platelets (means ± SD) (six experiments/group); 2 x 10^6 platelets were incubated for 3 hours at 37°C in the following way: 1) control platelets in their own plasma (1 ml); 2) oral contraceptive platelets in their own plasma; 3) control platelets in oral contraceptive plasma; and 4) oral contraceptive platelets in control plasma.

Significance: group 2 vs. 1 (unpaired t test)—p<0.05, t<0.01; group 3 vs. 1 (paired t test)—tp<0.025, t<0.01, ||p<0.05; group 4 vs. 2 (unpaired t test)—t<0.05.

Table 2. Influence on Platelet Aggregation of Plasma Peroxidized Free Fatty Acids from Oral Contraceptive-treated Animals

| Source of peroxidized free fatty acids | Aggregation (cm) |
|---------------------------------------|------------------|
|                                        | Thrombin         | Adenosine diphosphate |
| Control plasma                        | 6.7 ± 4.9        | 9.8 ± 4.4            |
| Oral contraceptive plasma             | 16.5 ± 8.5*      | 13.3 ± 5.4†          |

Values are means ± SD of eight to nine experiments/group. Significance (paired t test) (oral contraceptive vs. control plasma): *p<0.001; †p<0.025.

Table 3. Lipids, Malondialdehyde, and Conjugated Dienes in Plasma of Oral Contraceptive-treated Female Rats

| Lipid                  | None Treatment | Oral Contraceptives |
|------------------------|----------------|---------------------|
| Total lipids (g/L)     | 4.2 ± 0.87     | 1.79 ± 0.85*        |
| Free fatty acids (g/L) | 0.48 ± 0.09    | 0.44 ± 0.11         |
| Malondialdehyde (mL/L) | 0.64 ± 0.08    | 0.91 ± 0.26†        |
| Conjugated dienes (mol/g lipid x 10^-4) | 0.04 ± 0.01 | 0.08 ± 0.01*       |

Values are means ± SD of seven to 12 experiments/group. Significance (unpaired t test): *p<0.001; †p<0.02.

In plasma, it seems to explain the almost immediate effect of OC plasma in activating the response of platelets to thrombin-induced aggregation. By contrast, the response of platelets to ADP is not significantly increased by OC plasma. Unlike thrombin aggregation, ADP aggregation is dependent on prostaglandin synthesis. It seems to depend largely on the formation of platelet lysate (lactonolsterol, cholesterol) accumulation, partly regulated by the biosynthesis. As to the lipid biosynthesis, it is also increased in normal platelet incubated in OC plasma, but it requires a longer incubation period than the thrombin-induced aggregation does (180 vs. 4 minutes) to be significantly stimulated. Therefore, it seems that lipid peroxides, enhanced in plasma, probably precede and initiate the platelet abnormalities induced by OC administration, at least in rats.

Acknowledgments

We thank G. Pelon, Université Claude Bernard, for editing the manuscript and C. Benoit for skillful technical assistance.

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Index Terms: thrombosis • estrogen • antioxidants • platelet aggregation