Effects of Estradiol On Platelet Aggregation In Cerebral Microvessels of Mice

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SUMMARY Mice were implanted subcutaneously with a pellet containing 0.5 mg estradiol or with a placebo. Eight to 12 days later platelet aggregation was produced in pial arterioles by injuring their endothelium in vivo with a noxious light/dye stimulus. The time between the onset of the noxious stimulus and the appearance of platelet aggregates was significantly shortened (p < .02) by estradiol treatment in young (2 month old) mice. The same treatment had the opposite effect in 4-6 month old mice and significantly delayed the onset of aggregation (p = .01). When platelet rich plasma (PRP) was prepared, aggregation by sodium arachidonate was always inhibited in PRP from estradiol treated mice, irrespective of age. Estradiol treatment had no effect on aggregation induced ex vivo by ADP. Thus the enhanced aggregation observed in pial arterioles of young estradiol treated mice may not reflect direct effects of estradiol on the platelet itself. The data are discussed in light of the literature suggesting enhancement of ischemic vascular disease, including strokes, in patients receiving estrogens, and especially high doses of estrogens.

IN SPITE OF great interest in the possible adverse effects of estrogen on blood clotting or hemostatic mechanisms in humans,1-4 few pertinent in vivo experimental studies have been reported. Most studies have been in vitro or ex vivo investigations. A single in vivo report indicated that estradiol and related estrogens enhanced platelet aggregation in injured mesenteric microvessels of female mice.5 Others have shown that estradiol inhibits the ability of arachidonate to induce platelet thrombi in mouse pulmonary vessels. This difference in the platelet response within different vascular beds may indicate that platelet aggregation in vivo is a complex function of drug or hormone action directly on the platelet itself and indirectly on the platelet via drug effects on the metabolism of the vessel wall and/or adjacent tissue. Indeed we have shown that a drug tested in exactly the same way in 2 different microvascular beds in the same species, can produce opposite effects on platelet aggregation in one bed as opposed to the other.9, 10 Therefore, in order to draw conclusions concerning estradiol’s action on aggregation in cerebral vessels it is necessary to use the cerebral vasculature in an appropriate study. In view of the absence of any published experimental work concerning estradiol’s action on platelet aggregation in cerebral vessels, and in view of suggestions that estrogens may be a factor enhancing ischemic stroke2,3 we performed the investigation described below.

Methods

Platelet Aggregation in Vivo

Our methods have been published in great detail.9-13 Endothelial injury13 is produced by exposing the vessels in a microscopic field to filtered light from a mercury lamp via epillumination and Leitz Ultropak objectives.9, 11, 13 The filtered light is innocuous unless sodium fluorescein (0.8 ml of a 2% solution per 100g body weight) is intravenously injected. It is probable that the endothelial injury13 is initiated by free radicals generated when the dye is excited.14 We measure, in seconds, the latent period between application of light and dye, and the onset of the first recognizable adherent platelet aggregate in an arteriole in the microscopic field. The vessel is preselected arbitrarily from arterioles 30-60 μ in internal diameter, at the site of craniotomy.15 With our method of inducing aggregation it is easy to monitor aggregation because the aggregates fluoresce when appropriate barrier filters are used.9, 11 The method has enabled us to detect a variety of drug effects on platelet aggregation in cerebral surface vessels (pial vessels). Some of these effects are the anti-aggregatory action of cyclooxygenase inhibitors,9, 16
and of prostacyclin,\textsuperscript{17} and the proaggregatory effects of a prostacyclin synthesis inhibitor.\textsuperscript{12}

Mice of the ICR strain (Dominion Laboratories, Virginia) were used. Vessels on the surface of the brain (pial vessels) were exposed by craniotomy in urethane anesthetized mice, as previously described.\textsuperscript{15, 18} The surface was kept moist by a drop of Elliott's solution\textsuperscript{15} held in the minute space between the cerebral surface and the immersion attachment on the 22X objective lens. The mice were maintained at 37°C. At the end of each experiment, arterial levels of CO\textsubscript{2}, O\textsubscript{2}, and pH were obtained from a 100 \( \mu \)L sample. Mean values for all studies were CO\textsubscript{2} = 27 ± 4, O\textsubscript{2} = 100 ± 8, pH = 7.43 ± .05. There were no significant differences between values in hormone treated and placebo treated mice so these data will not be referred to later.

We also measured center line red cell (RBC) velocity using an IPM (LaMesa, CA) two slit velocimeter.\textsuperscript{19} Shear rates at the vessel wall were calculated from the velocity values and the internal diameter of the arteriole. The diameter was measured with an ocular micrometer. The shear rates were of interest because we had shown a direct relationship between these values and the latency of aggregation.\textsuperscript{19}

\textbf{Platelet Aggregation \textit{ex Vivo}}

Platelet rich plasma (PRP) was prepared from mouse blood as previously described\textsuperscript{20, 21} using centrifugation at 126Xg to separate the red cells from the overlying PRP. Generally blood from two mice was pooled to make PRP for each tested sample. Platelet counts in PRP were adjusted to 400,000/\( \mu \)L with platelet poor plasma (PPP) prepared from centrifuging blood at 1400 Xg after first decanting the PRP. Aggregation of a 250 \( \mu \)L aliquot of PRP was monitored as a change in optical density in a Chronolog Lumi aggregometer at 37°C. The aggregating agents used were ADP (Sigma) 0.5 \( \mu \)M final concentration, and sodium arachidonate (Nuchek 98% pure).

The release reaction of platelets was monitored in PRP by detecting released ATP using the luciferin-luciferase reaction in the aggregometer.\textsuperscript{22, 23} The final concentration of the luciferin-luciferase reagent (Chronolog, Haverton PA) was 1.6 mg/ml. The system was calibrated by adding known amounts of ATP to PRP.

\textbf{Hormone Treatment}

A pellet (Innovative Research, Rockville, MD) containing 0.5 mg of 17-beta-estradiol was implanted subcutaneously 8–19 days prior to studying platelet aggregation. Several studies were carried out. In each, a group of placebo implanted mice was studied simultaneously with the hormone treated mice. The placebo contained the same filler as the hormone tablet. The placebo animals arrived in the animal care quarters at the same time as the parallel hormone treated group. A hormone treated mouse was alternated with a placebo treated animal on each day of each study. This assured us that comparisons were made between groups of mice that were as similar as possible in every respect except hormone treatment.

Data provided by the manufacturer of the pellets indicates that they markedly elevate hormone levels in mice by 7 days after implantation and that these levels remain constant at the elevated level for over 4 weeks. We measured serum estradiol levels using radioimmunoassay (Columbia Diagnostics, Springfield, VA) and confirmed this. Placebo treated mice had less than 50 pg/ml of estradiol but levels in estradiol treated mice were approximately 1000 pg/ml throughout the test period. For example 950 ± 497 on day 10, 933 ± 466 on day 12, and 1003 ± 617 on day 19 (M ± SD, N = 10 in each group).

\textbf{Statistical Techniques}

Estradiol treated groups were compared with placebo treated groups using two-way analysis of variance (ANOVA).

\textbf{Results}

\textbf{In Vivo}

We initially looked at the effect of estradiol in two completely independent studies of 2 month old mice. First, aggregation in arterioles of hormone treated males was compared with that in arterioles of contemporary placebo treated males. The arteriolar diameter was virtually identical in the two groups (33 ± 2\( \mu \)M vs 32 ± 2\( \mu \)M ± SD). Later we compared aggregation in pial arterioles of estradiol treated females with that of placebo treated females. Again arteriolar diameter was the same in the two groups (34 ± 4\( \mu \)M vs 35 ± 3\( \mu \)M). In both the study of males and the study of females, following the noxious stimulus, aggregation occurred more rapidly in the estradiol treated mice (\( p < .02 \) ANOVA, table 1). The effect of estradiol on aggregation latency was not related to changes in shear rate of blood through the arterioles since the shear rate in estradiol males was like that in placebo males (1599 ± 566 vs 1480 ± 298 reciprocal sec., M ± SD), and the shear rate in estradiol females was like that in placebo females (1920 ± 890 vs 1969 ± 600).

We then looked at the effect of estradiol in older (4–6 month old) mice. Again two independent studies were carried out; one in hormone treated males vs contemporary placebo treated males, and one in horm-

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
& \textbf{Male} & \textbf{Female} \\
\hline
\textbf{Estradiol}\textsuperscript{*} & 64 ± 14 (N = 10) & 63 ± 21 (N = 10) \\
\textbf{Placebo} & 83 ± 21 (N = 10) & 76 ± 23 (N = 10) \\
\hline
\end{tabular}
\caption{Estradiol Enhances Platelet Aggregation in Pial Arterioles of 2 Month Old Mice}
\end{table}

\textsuperscript{*}Effect of treatment significant by analysis of variance (\( p < .02 \)).

A tablet containing 0.5 mg estradiol or a placebo tablet was implanted subcutaneously in each mouse. Eight to 12 days later, we determined the time required for the noxious stimulus to elicit platelet aggregation, \textit{in vivo}. The data are expressed as mean seconds ± standard deviation. Estradiol significantly shortened the time to aggregation.
mone treated females vs contemporary placebo treated females. Arteriolar diameter was 33 ± 3μ in each group of males, 34 ± 5μ in estradiol females and 34 ± 4μ in placebo females. Again there was no difference in shear rate between each estradiol treated group and its respective placebo control (1228 ± 213 vs 1001 ± 206 reciprocal sec, M ± SD, in the males and 1099 ± 294 vs 1041 ± 248 in the females). Again estradiol altered platelet aggregation, but the effect was opposite that noted in younger mice. That is estradiol treated mice displayed longer aggregation latencies (delayed or inhibited aggregation) when compared with their respective placebo controls (p < .02 ANOVA, table 2).

In Vitro

Because we found opposite effects of estradiol in vivo, in mice of different ages, it was of interest to see whether the in vivo effects were mirrored by in vitro responses. Would platelets taken from young estradiol treated mice show enhanced aggregation, while platelets from older estradiol treated mice showed impaired aggregation? The answer is no. In fact there were striking disparities between in vivo and in vitro results.

Firstly, platelets from female mice were less sensitive to arachidonate than platelets from males; a sex difference not reflected by the sex-neutral in vivo data. The decreased sensitivity of female platelets forced us to test them at higher concentration (0.5 mM) of sodium arachidonate than the concentration (0.25 mM) used to test male platelets. Second, as shown in table 3, platelets from estradiol treated male mice were significantly less aggregable than platelets from male controls. Moreover they were less aggregable even in the young mice, in contrast to the enhanced aggregation seen in vivo after estradiol treatment of young males. ATP secretion mirrored the aggregation (0.50 ± 0.90 μM in young estradiol males vs 2.90 ± 2.60 in young placebo males and 0.30 ± 0.50 vs 1.50 ± 1.80 in older estradiol males vs older placebo males, M ± SD, p < .01 by ANOVA.). Platelets from estradiol treated females also showed significantly suppressed aggregation in vitro, in both young and older mice (table 4). Again the results with respect to aggregation, were mirrored by the data concerning ATP secretion by the aggregating platelets (0.20 ± 0.20 in young estradiol females vs 1.40 ± 2.90 in young placebo females, and 1.0 ± 1.9 vs 4.5 ± 2.6 in older estradiol treated females vs older placebo treated females, μMATP, M ± SD p < .01 by ANOVA). Thus, at least when arachidonate was used as an in vitro stimulus, there was no enhancement of aggregation in younger mice, males or females, as had been seen in vivo following estradiol treatment.

When 0.5 μM ADP was used as the stimulus for aggregation, no differences were seen between platelets from estradiol and placebo treated mice. Maximal aggregation expressed as percent light transmitted (M ± SD) was 67 ± 16 in platelets from 2 month old males, estradiol treated, vs 72 ± 8 in platelets from 2 month old males placebo treated. Aggregation to ADP was 63 ± 12 in 4 month old males estradiol treated vs 58 ± 21 in 4 month old placebo treated males. Similarly there was no difference in aggregation between hormone treated two month old females (58 ± 13) and their placebo treated controls (66 ± 12) or between hormone treated 4 month old females (67 ± 11) and their placebo treated controls (64 ± 17). Since hormone treatment failed to influence ex vivo responses to ADP, we see again a failure of ex vivo results to parallel the in vivo observations.

Discussion

Two findings emerge from the data. First, estradiol alters the time required for a constant noxious stimulus to produce adherent platelet aggregates over a region of injured endothelium in the cerebral microcirculation. Second, the direction of the estradiol effect de-

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**Table 2** Estradiol Inhibits Platelet Aggregation in Pial Arterioles of Mice 4–6 Months Old

<table>
<thead>
<tr>
<th></th>
<th>Seconds required to elicit aggregation</th>
</tr>
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<tbody>
<tr>
<td>Estradiol*</td>
<td>70 ± 29 (N = 10) 71 ± 22 (N = 10)</td>
</tr>
<tr>
<td>Placebo</td>
<td>44 ± 11 (N = 10) 64 ± 12 (N = 10)</td>
</tr>
</tbody>
</table>

*Effect of treatment significant by analysis of variance (p < .01).

The procedure is the same as that described for table one, but the mice were 4–6 months old, rather than 2 months old at the time of implant. Estradiol now significantly lengthened, rather than shortened, the time (mean seconds ± standard deviation) required for the noxious stimulus to elicit aggregation.

**Table 3** Estradiol Given to Male Mice Inhibits Platelet Aggregation Ex Vivo to 0.25 mM Arachidonate

<table>
<thead>
<tr>
<th>Aggregation (% transmitted, M ± SD)</th>
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</thead>
<tbody>
<tr>
<td>Estradiol*</td>
</tr>
<tr>
<td>Placebo</td>
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</table>

*Effect of treatment significant by analysis of variance (p < .001).

Pellets of either estradiol or placebo were implanted as in the in vivo studies, but now platelets were harvested in platelet rich plasma and stimulated ex vivo by arachidonate in an aggregometer. The table displays maximal aggregation produced by 0.25 mM arachidonate, expressed as % transmitted light (M ± SD) in the aggregometer. Estradiol significantly diminished the response.

**Table 4** Estradiol Given to Female Mice Inhibits Platelet Aggregation Ex Vivo to 0.5 mM Arachidonate

<table>
<thead>
<tr>
<th>Aggregation (% transmitted, M ± SD)</th>
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</thead>
<tbody>
<tr>
<td>Estradiol*</td>
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<tr>
<td>Placebo</td>
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</table>

*Effect of treatment significant by analysis of variance (p < .01).

Pellets were implanted as before, and studied ex vivo as in table three. But here female mice were used, and their platelets required a higher concentration of arachidonate (0.5 mM) to reliably elicit aggregation. As with platelets from males, estradiol treatment of the animals significantly depressed the ex vivo response.
pends on the age of the mice receiving the estradiol. Third, the in vivo effect of estradiol was not explained by the behavior of the platelets examined ex vivo in PRP. These findings will be discussed in turn.

In mice 2 months old, the induction of adherent aggregates was enhanced in pial arterioles. On the other hand, in the pial arterioles of older animals the induction of aggregates was inhibited by estradiol. There are several possible reasons for the pro or antiaggregatory effects of estradiol. Estradiol may alter the platelet directly. Estradiol may alter the composition of the plasma. Estradiol may alter the release of pro or antiaggregatory substances by vessel or surrounding tissue. Since the ex vivo data with platelet rich plasma from two month old mice demonstrated that platelet aggregation by ADP was unaffected by estradiol treatment and platelet aggregation by arachidonate was suppressed by estradiol treatment, the proaggregatory effect of estradiol seen in vivo in two month old mice is not likely to be mediated by an effect of estradiol on the platelet or plasma. However it is possible that estradiol reduces the production or release of proaggregants by injured endothelial cells in pial arterioles of young mice. Experiments will have to be designed to test this possibility. Whatever the nature of the proaggregatory factors at work following estradiol treatment in the two month old mice, the strength of these forces apparently diminishes in older animals. We then observe delayed or inhibited aggregation in the older estradiol treated mice. The latter effect may be a reflection of the direct antiaggregatory effect of estradiol treatment on the platelet, since aggregation, at least to arachidonate, was inhibited ex vivo as well. The two month old mice and the older animals were all sexually mature. From the information at hand it is not possible for us to relate the response of the older mice to any particular consequence of their longer period as sexually mature animals.

Decreased responses to arachidonate of platelets from estradiol treated mice suggests decreased cyclooxygenase activity in the platelets, since arachidonate is the substrate for that activity. Moreover, in the rat, cyclooxygenase activity has been reported suppressed in females by at least two groups of investigators. This also suggests a depression of cyclooxygenase activity, in rodents, by estrogens. Consonant with this suggestion is our finding that higher concentrations of arachidonate had to be used to elicit aggregation in platelets from female mice as compared with males. This is also in agreement with data reported by others from rats and quite different from human data, where females demonstrate enhanced aggregation to a variety of agents. Some workers have concluded that ex vivo sex related differences in humans were really due to hematocrit differences resulting in lower anticoagulant concentrations in female plasma. Others could not account for increased aggregation in PRP from females on this basis. Our male mice did not show significantly different HCt from our females.

A direct effect of estrogens on platelets would not be surprising since estrogen receptors have been demonstrated in these cells. However there are other possibilities. Very high levels of estradiol may elicit a variety of chemical changes in turn could alter the physical and/or chemical composition of the platelet, producing diminished aggregation both in vivo and ex vivo.

Finally, our data should not be extrapolated to vascular beds outside the brain. Conclusions concerning drug effects on aggregation in a given vascular bed must rest on studies of that particular bed. While this investigation was stimulated by reports that estrogens increase the incidence of cerebrovascular accidents in humans our results neither confirm nor deny the hypothesis that such an adverse side effect may be related to increased platelet aggregation in cerebral vessels. Our results show that estradiol enhanced in vivo aggregation only in very young mice, while in older mice, estradiol treatment was associated with impaired aggregation. Moreover, the levels of estradiol achieved in our mice are considerably higher than those produced by oral contraceptives in humans and approach those of pregnancy. The levels do, however, resemble those seen in humans given high dose estrogen for treatment of prostatic carcinoma and myocardial infarction. The latter high dose studies had to be discontinued because of an increased incidence of thromboembolic events. It may be that our data from the two month old mice is relevant to the increased thromboembolic incidence seen in humans given high dose estrogen. However extrapolation from mouse to man is exceedingly difficult and, in the present case, cannot be sustained without a great deal of additional study.

References
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