Combined Endothelial Nitric Oxide Synthase Upregulation and Caveolin-1 Downregulation Decrease Leukocyte Adhesion in Pial Venules of Ovariectomized Female Rats

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Background and Purpose—We recently found that chronic estrogen depletion enhances leukocyte adhesion in pial venules in the female rat, while estrogen repletion decreases it. Estrogen-associated repression of inflammation may be due to upregulation of the endothelial isoform of nitric oxide synthase (eNOS) and concomitant downregulation of the endogenous inhibitor of eNOS, caveolin-1 (CAV-1). In this study we examined the effects of estrogen-independent eNOS upregulation (via simvastatin) and/or CAV-1 downregulation (antisense) on pial venular leukocyte adhesion in ovariectomized (OVX) rats.

Methods—Intact and OVX rats were prepared with closed cranial windows. Adherent rhodamine 6G–labeled leukocytes were viewed by intravital microscopy. To demonstrate the importance of pial venular eNOS in the resistance to leukocyte adhesion, intact female rats were treated with a nonselective (N\textsuperscript{G}-nitro-L-arginine) or a neuronal NOS–selective (7-nitroindazole) inhibitor. In OVX females, leukocyte adhesion was compared in the following groups: (1) untreated; (2) treated with simvastatin; (3) treated with simvastatin plus CAV-1 antisense; (4) treated with simvastatin plus CAV-1 missense; (5) treated with CAV-1 antisense; and (6) treated with CAV-1 missense.

Results—In intact females, pial venular leukocyte adhesion was increased when total NOS activity, but not neuronal NOS activity alone, was blocked. In OVX rats, basal leukocyte adhesion, measured as the percentage of venular area occupied by adherent leukocytes, was attenuated (by \(\approx 60\%\)) only in the presence of combined simvastatin plus CAV-1 antisense treatment.

Conclusions—Present findings demonstrate that eNOS-derived NO plays an important role in limiting cerebral venular leukocyte adhesion in female rats. These data also suggest that simvastatin-induced upregulation of eNOS expression in OVX rats will not restore eNOS function, as measured by decreased leukocyte adhesion, unless CAV-1 levels are reduced as well. (Stroke. 2002;33:613-616.)

Key Words: cell adhesion ■ cerebral circulation ■ estrogens ■ nitric oxide ■ simvastatin ■ rats

We previously reported that estrogen depletion increases, while estrogen replacement decreases, leukocyte adhesion in the cerebral circulation of female rats during resting conditions and after transient forebrain ischemia.\(^1,2\) This effect may be due, at least in part, to the capacity of estrogen to upregulate endothelial nitric oxide synthase (eNOS) expression and increase NO generation in endothelial cells.\(^3,4\) NO produced in the endothelium is well known for its antiadhesive properties.\(^5,6\) Recently, in a preliminary study, we attempted to decrease leukocyte adhesion in ovariectomized (OVX) female rats via an estrogen-independent upregulation of brain eNOS expression. Thus, experiments using chronic treatment of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin were performed. Simvastatin has been shown to stabilize ENOS mRNA in rodents, resulting in a cholesterol-independent increased expression of eNOS protein.\(^7,8\) Surprisingly, despite increased eNOS expression, leukocyte adhesion remained unchanged compared with vehicle-treated counterparts.\(^9\) In an effort to explain this result, we relied on data from 2 recently published studies from our laboratory. In the first study estrogen was shown to not only upregulate eNOS expression and function but also to downregulate the expression of its endogenous inhibitor, caveolin-1 (CAV-1).\(^4\) In the second study it was found that upregulation of eNOS in OVX rats, via simvastatin, was ineffective in restoring agonist (ie, acetylcholine)-stimulated eNOS activation in pial arterioles, unless combined with a concomitant downregulation of CAV-1 expression.\(^10\) Thus, it is possible that upregulation of eNOS expression alone will not necessarily translate into decreased leukocyte adhesion in pial venules, unless CAV-1 expression is downregulated as well.

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In the present study experiments were undertaken to test the hypothesis that upregulation of eNOS expression, using simvastatin treatments, needs to be combined with downregulation of CAV-1 expression, using antisense oligonucleotide techniques, before one can reverse the enhancement of cerebral venular leukocyte adhesion seen in OVX females. In parallel evaluations in which intact females were used, a series of experiments was performed to confirm the importance of pial venular eNOS in the resistance to leukocyte adhesion in our model, using applications of nonselective (N^3-nitro-L-arginine [L-NNA]) and neuronal NOS (nNOS)—selective (7-nitroindazole [7-NI]) NOS inhibitors.

Materials and Methods
The Institutional Animal Care and Use Committee approved the study protocol. Forty-one adult female Sprague-Dawley rats (Charles River, Wilmington, Mass) weighing 300 to 400 g were used. Rats were divided in 2 groups: intact females (n=11, used for the NOS inhibition studies) and OVX females (n=30). The supplier performed ovariectomies 4 to 6 weeks before the study. In the NOS inhibitor experiments, the intact female rats were further subdivided into 3 groups: treated with 7-NI (selective nNOS blocker), treated with L-NNA, and time control. The rats were anesthetized with halothane, tracheostomized, paralyzed with curaret, and ventilated with 0.8% halothane in 70% N₂/O₃/O₂. Femoral arterial and venous catheters were placed for monitoring of mean arterial blood pressure and arterial blood gases and for drug infusion, respectively. Rats were secured in a head holder in the prone position to facilitate placement of a closed cranial window. Details of this procedure are provided in previous publications.11,12 Briefly, a 10-mm-diameter craniotomy was performed over the skull midline, the underlying dura was carefully removed, and an 11-mm-diameter acrylic window, outfitted with 3 ports for inflow, outflow, and intracranial pressure monitoring, was placed and fixed to the skull with cyanoacrylate gel. After window placement, halothane was discontinued, and a bolus of fentanyl was given (10 µg/kg IV), followed by a maintenance dose of fentanyl 25 µg/kg per hour IV and ventilation with 70% N₂/O₃/O₂. These conditions were maintained throughout the study. Cannulas were inserted in the ports, and the space under the window was filled with artificial cerebrospinal fluid, which was suffused at a rate of 0.5 mL/min and maintained at a temperature of 37°C. PO₂=40 to 45 mm Hg, PO₃=50 to 60 mm Hg, and pH≈7.35. Intracranial pressure was maintained at 5 to 10 mm Hg by adjusting the height of the outflow cannula. Mean arterial blood pressure was continuously monitored, and rectal temperature was servo-controlled at 37°C. Arterial blood samples were taken for measurement of pH, Po₂, and Po₃. Those analyses were performed on an ABG 520 Blood Gas System (Radiometer).

Pial venules were viewed through a microscope (Nikon) equipped with a color video camera (Sony, Fryer Co Inc). An epi-illumination system with a mercury lamp was used. Pial venules were localized and displayed on a video monitor. Leukocytes were labeled with rhodamine 6G (200 µg/mL in 0.9% saline) given initially as an intravenous bolus (1 mL) and followed by continuous infusion at a rate of 1 mL/h.1,2,13 A rhodamine filter set was inserted into the light path, and a videotape record of baseline leukocyte dynamics was obtained before the administration of the appropriate NOS inhibitor (or vehicle). 7-NI (40 µg/kg IP) or L-NNA (1 mmol/L solution suffused continuously under the window) was given, and leukocyte dynamics were video recorded after 30, 60, and 120 minutes. In all cases, illumination was limited to 60 seconds at a time to avoid photo quenching. Subsequent analysis of leukocyte adhesion was performed by capturing multiple frames of taped images in a computer and measuring the percentage of the venular area occupied by adhering leukocytes with the use of the Image Pro Plus analysis system (Media Cybernetics). In the time control group, no drugs were given, artificial cerebrospinal fluid was suffused continuously, and leukocyte dynamics were recorded at baseline and after 30, 60, and 120 minutes.

For the OVX animals, the experimental design was divided into 2 stages. In the first stage all rats were prepared with closed cranial windows, as described in a recent publication,10 and returned to their cages. The second stage commenced 1 day after window placement, when the animals were reanesthetized (fentanyl/IN₂O₃), followed by placement of femoral venous and arterial catheters and reexposure of the cranial windows.10 Leukocytes were labeled with rhodamine 6G, and leukocyte adhesion during resting conditions was compared in the following OVX groups: (1) untreated; (2) treated with simvastatin; (3) treated with simvastatin plus CAV-1 antisense; (4) treated with simvastatin plus CAV-1 missense; (5) treated with CAV-1 antisense; and (6) treated with CAV-1 missense. In the simvastatin-treated groups, simvastatin was administered over 2 weeks at a dose of 20 mg/kg per day SC. The CAV-1 antisense (5'-TTTACC CCCAGACAT-3') or missense (5'-CAATCGGTAAACC-3') was introduced into the space under the cranial window (25 µg in ≈300 µL of artificial cerebrospinal fluid) 24 hours before leukocyte adhesion measurement (ie, during the first experimental stage). The rationale for selection of these oligonucleotide sequences was provided in a previous publication.10 The effect of a lower dose of CAV-1 antisense was also tested (10 µg), creating variations of the aforementioned groups 3 and 5.

Statistical analyses were performed with paired t test and 1-way ANOVA with a post hoc Tukey test for multiple comparison procedures. A level of P<0.05 was considered significant in all statistical tests. Values are presented as mean±SE. The oligonucleotides were obtained from Sigma Genosys in the unmodified phosphodiester form. All other drugs and chemicals were obtained from Sigma, unless otherwise stated.

Results
The physiological variables in all groups and experiments were within normal limits. There were no significant differences in pH, Po₂, or mean arterial blood pressure in any of the groups studied and over the course of each experiment (data not shown). The range of diameters for the pial venules studied was 35 to 70 µm. In the NOS inhibition experiments, the percent venular area occupied by adherent leukocytes at baseline was 6.2±2.1% (7-NI), 4.8±0.6% (L-NNA), and 4.2±0.7% (control). Leukocyte adhesion was considerably elevated in the L-NNA–treated group. The percent venular area occupied by adherent leukocytes in that group rose to 9.3±0.7% and 12.2±1.4% after 1 and 2 hours, respectively (Figure 1). In contrast, leukocyte adhesion in the 7-NI–treated group and time controls showed similar and rather modest
changes, increasing to 8.4 ± 1.3% and 6.8 ± 1.8% after 1 hour and 7.9 ± 1.4% and 6.9 ± 1.6% after 2 hours, respectively (Figure 1). This demonstrates the importance of pial venular eNOS-derived NO in the resistance to leukocyte adhesion in our model.

In OVX animals, leukocyte adhesion during resting conditions was significantly decreased (~60%) only in the group treated with simvastatin plus CAV-1 antisense. The percentage of the venular area occupied by adherent leukocytes in this group was 4.4 ± 0.3% compared with 10.4 ± 1.5%, 11.9 ± 1.2%, 12.7 ± 1.1%, 10.9 ± 1.2%, and 12.4 ± 1.2% in the untreated OVX group and groups treated with simvastatin, simvastatin plus missense, CAV-1 antisense, and CAV-1 missense, respectively (Figure 2). The results of experiments using the 2 different doses of CAV-1 antisense (10 and 25 μg) alone and combined with simvastatin treatment are reported as single groups because they produced very similar effects on leukocyte adhesion. Note also that intact females treated for 2 hours with L-NNA (Figure 1) show levels of leukocyte adhesion very similar to those seen in untreated OVX animals (Figure 2).

Discussion
The key findings of the present study can be summarized as follows. First, inhibition of eNOS, but not nNOS, significantly increases basal leukocyte adhesion in intact females. Second, the enhanced baseline leukocyte adhesion observed in OVX (chronically estrogen-depleted) females was reduced to levels seen in intact females only in the presence of combined eNOS upregulation and CAV-1 downregulation, but not when those manipulations were applied separately. The first observation establishes the important role of eNOS-derived NO in preventing excessive leukocyte adhesion in cerebral vessels of female rats. The second finding not only corroborates previous results from our laboratory, showing enhanced leukocyte/inflammatory activity in cerebral venules of estrogen-depleted females,1,2 but also provides a mecha-

nistic link between the increased leukocyte adhesion and the repression of eNOS function4,10 that has been observed in these animals. The latter results, therefore, indicate that, at least in pial vessels, statin-induced upregulation of eNOS expression in OVX rats will not restore eNOS function, as measured by decreased leukocyte adhesion, unless the elevated levels of the eNOS inhibitory protein, CAV-1, are reduced as well. Those findings further imply that CAV-1 downregulation, by itself, is ineffective in restoring eNOS activity if eNOS abundance is too low. A similar result was obtained by us10 when using another end point for assessing eNOS function, ie, agonist (acetylcholine)-induced, eNOS-dependent cerebral vasodilation.

In a recent publication, we established the validity of the present model with respect to the ability of chronic simvastatin administration to increase eNOS expression in pial vessels of OVX rats without affecting CAV-1 expression and with respect to the efficacy of CAV-1 antisense treatments.10 In contrast, Feron et al14 reported that prolonged exposure to statins can increase eNOS activity, in isolated bovine aortic endothelial cells, via reductions in the levels of CAV-1 and its binding to eNOS. No specific explanation for this dissimilarity in experimental results can be provided at this time. Nevertheless, it merits consideration that differences in statin actions on endothelial CAV-1 expression and eNOS/CAV-1 interactions related to dose, vascular bed, species, and especially model (ie, in vivo versus in vitro) may account for that disagreement.

That NO may play an antiadhesive role in the cerebral circulation was shown in a report by Hudetz et al. In that study, administration of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) just before reperfusion in rats subjected to forebrain ischemia was associated with an increase in the posts ischemic levels of leukocyte adhesion. Monitoring only resting leukocyte behavior, Gidday et al16 reported an increase in pial venular leukocyte adhesion in newborn pigs in the presence of acute NOS inhibition. In contrast, other investigators have reported no changes in basal leukocyte adhesion in rat pial venules after NOS inhibitor administration,13,17 although in 1 of those studies13 the proadhesive actions of leukotriene B4 were enhanced in the presence of NOS inhibition. Unfortunately, there is no experimental evidence that can provide any definitive clues to explain these seemingly contradictory findings. An intriguing prospect relates to sex, insofar as males were used in those earlier investigations. Thus, perhaps because of the seemingly greater levels of cerebral eNOS expression and activity in females versus males (reviewed in Reference 6), females may have developed a greater reliance on eNOS-derived NO in resisting cerebral inflammatory activity than males, while males may make greater use of “alternative” counterinflammatory mechanisms. Clearly, further study is required.

Another possibility relates to the duration of the reduction in eNOS activity. Previous studies on the brain vasculature involved acute NOS blockade paradigms, whereas ovarectomy is essentially a model of chronic eNOS repression. In the peripheral circulation, chronic inhibition of basal release of NO induces adhesion molecule expression, indicative of a tonic anti-inflammatory role of NO. Related to this,

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it was recently shown that increasing eNOS expression (via liposome transfection) in donor hearts reduced coronary leukocyte activity, relative to controls, after transplantation. On the other hand, in cerebral vessels there is a paucity of information regarding the effects of chronic reductions in eNOS activity on leukocyte adhesion. However, in a recent preliminary study, we found that chronic NOS inhibition did not increase adhesion molecule (ie, intercellular adhesion molecule-1) expression in parenchymal microvessels under resting conditions. Nevertheless, one could not conclude that baseline leukocyte adhesion was also unaffected because this was not monitored. Additionally, these experiments were performed on male rats. Thus, the possibility of sex-related differences remains open.

In conclusion, present findings indicate that eNOS-derived NO plays an important role in limiting cerebral venular leukocyte adhesion in female rats. The increase in leukocyte adhesion in chronically estrogen-depleted females seen in the present and previous investigations appears to relate, at least in part, to a diminished cerebrovascular eNOS activity. However, it should be emphasized, as discussed in earlier reports, that the influence of estrogen on cerebral leukocyte adhesion probably involves eNOS-independent actions as well. The documented repression of eNOS function likely relates to a downregulation of eNOS expression combined with an upregulation of the endogenous NOS inhibitor CAV-1, since reducing leukocyte adhesion to levels seen in “estrogen-normal” females required a concomitant increase in eNOS and decrease in CAV-1 expression in cerebral venules.

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References

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