Argatroban Attenuates Leukocyte– and Platelet–Endothelial Cell Interactions After Transient Retinal Ischemia

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Background and Purpose—Argatroban, a direct thrombin inhibitor, has been shown to reduce neural injury after transient cerebral ischemia. It has also been reported that this neuroprotective effect results from an anticoagulant function. This study was designed to evaluate quantitatively the inhibitory effects of argatroban on leukocyte– and platelet–endothelial cell interactions after transient retinal ischemia.

Methods—Retinal ischemia was induced for 60 minutes in male Long-Evans rats by temporary ligation of the optic sheath (n = 342). Argatroban was administered just after induction of ischemia. Leukocyte and platelet behavior in the retinal microcirculation was then evaluated in vivo with scanning laser ophthalmoscopy. The expression of P-selectin and intracellular adhesion molecule-1 (ICAM-1) was evaluated by reverse transcription–polymerase chain reaction. After 10 days of reperfusion, ischemia-induced retinal damage was evaluated histologically.

Results—Treatment with argatroban suppressed leukocyte–endothelial cell interactions; the maximum numbers of rolling and accumulated leukocytes were reduced by 90.1% (P < 0.05) and 58.7% (P < 0.05), respectively, at 12 hours after reperfusion. Treatment with argatroban also suppressed platelet–endothelial cell interactions; the maximum numbers of rolling and adhering platelets were reduced by 91.8% (P < 0.01) and 78.9% (P < 0.01), respectively, at 12 hours after reperfusion. The expression of P-selectin and ICAM-1 mRNA was suppressed significantly in the argatroban-treated retinas (P < 0.01). Histologic examination demonstrated the protective effect of argatroban on ischemia-induced retinal damage (P < 0.01).

Conclusions—Argatroban treatment suppressed leukocyte– and platelet–endothelial cell interactions after transient retinal ischemia. This inhibitory effect on postischemic blood cell–endothelial cell interactions might partially contribute to its neuroprotective effects. (Stroke. 2003;34:2043-2049.)

Key Words: ischemia • leukocytes • platelets • retina • rheology
inflammatory mediators \(^{20,21}\) and recruit leukocytes to ischemic regions through the expression of adhesion molecules on their surfaces or by the production of cytokines. \(^{22,23}\) Argatroban prevents coagulation by inhibiting platelet–platelet interaction and fibrin formation, events that could otherwise lead to inhibition of postischemic thrombus formation. \(^{4,5}\) Argatroban might also prevent inflammation by inhibiting platelet–endothelial cell interactions.

We have developed in vivo methods to quantitatively evaluate leukocyte– \(^{10,24,25}\) and platelet– \(^{18,19}\) endothelial cell interactions in the rat retina. The optic media, which consists of the cornea, lens, vitreous, and retina, are so transparent that the retinal microcirculation can be observed noninvasively in vivo. The retina is part of the central nervous system, and the properties of endothelial cells and neural cells in the retina are similar to those in the cerebrum. \(^{26,27}\) Therefore, investigation of leukocyte and platelet dynamics in postischemic retina might be extrapolated to leukocyte or platelet involvement in postischemic brain injury. The purpose of this study was to evaluate quantitatively the inhibitory effects of argatroban on leukocyte– and platelet–endothelial cell interactions in vivo in postischemic retina and to study the therapeutic efficacy of argatroban on retinal injury after transient ischemia.

## Materials and Methods

### Animal Model

Induction of transient retinal ischemia was reported previously. \(^{10,28}\) Male pigmented Long-Evans rats (200 to 220 g; \(n = 542\) ) were anesthetized with xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. After a lateral conjunctival peritomy and incision of the pars plana vitrea, the eye was exposed by blunt dissection, and a 6-0 nylon suture was passed around the optic sheath and tightened until blood flow ceased in all retinal vessels. The absence of perfusion for a 60-minute period was confirmed with the use of an operating microscope, after which the suture was removed.

Argatroban (obtained from Mitsubishi Pharma Corporation/Daiichi Pharmaceutical) was dissolved in 0.9% NaCl solution containing HCl to prepare concentrations of 30 mg/mL. The vehicle was 0.9% NaCl solution containing HCl. To prepare 3 mg/mL argatroban solution or vehicle, was infused by way of an osmotic pump (10 \(mL/h\) ) that was implanted intraperitoneally immediately after ischemia induction. To investigate the dose dependence of argatroban, 2 additional doses of argatroban (3 and 0.3 mg/mL) were used.

Peripheral blood specimens were collected to count the number of leukocytes and platelets with use of a hematologic analyzer (ERMA) and to measure the PT and APTT at various reperfusion points. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Leukocyte–Endothelial Cell Interactions

To evaluate leukocyte–endothelial cell interactions after transient retinal ischemia, we used acridine orange (AO) digital fluorography, which has been described in detail elsewhere. \(^{10,24,25}\) In brief, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments), coupled with a computer-assisted image analysis system, made continuous, high-resolution images of the fundus stained by metachromatic fluorochrome AO (Wako Pure Chemicals). \(^{28}\) For further analysis, the obtained images were recorded on an S-VHS videotape at a rate of 30 frames/s.

AO digital fluorography was performed at 4, 12, 24, 48, and 72 hours after reperfusion. Six different rats in the argatroban-treated and vehicle-treated groups were used at each time point. Six

nonoperated rats served as controls. Immediately before AO digital fluorography, the rats were anesthetized and the pupils were dilated. A contact lens was used to retain corneal clarity throughout the experiment. Arterial blood pressure was monitored with a blood pressure analyzer (IITC). AO (0.1% solution in saline) was injected continuously through the tail vein catheter for 1 minute at a rate of 1 mL/min. Rolling leukocytes were defined as those that moved at a velocity slower than that of free-flowing leukocytes. The number of rolling leukocytes was calculated from the number of cells per minute crossing a fixed area of the vessel at a distance of 2 disk diameters from the edge of the optic disk. The number of rolling leukocytes was defined as the total number of rolling leukocytes along all major veins.

At 30 minutes after the injection of AO, the fundus was observed again to evaluate leukocyte accumulation in the retinal microcirculation. The number of fluorescent dots in the retina within 8 areas of 100² pixels square at a distance of 2 disk diameters from the edge of the optic disk was counted. The average number of individual areas was used as the number of leukocytes accumulated in the retinal microcirculation for each rat.

### Platelet–Endothelial Cell Interactions

To evaluate platelet–endothelial cell interactions after transient retinal ischemia, we used fluorescently labeled platelets, a technique that has been described in detail elsewhere. \(^{18,19}\) In brief, platelet samples were harvested from donor rats and stained with carboxyfluorescein diacetate succinimidy ester (Molecular Probes). After each rat was anesthetized, 6 × 10⁵ fluorescently labeled platelets were infused into the tail vein catheter. Platelet behavior in the retinal microcirculation was then observed with an SLO and recorded for further analysis.

Platelet behavior in the retinal microcirculation was evaluated at 4, 12, 24, and 48 hours after reperfusion in both argatroban-treated and vehicle-treated groups. Six different rats were used at each time point. Rolling platelets were defined as those that moved at a velocity slower than that of free-flowing platelets. The number of rolling platelets in each major retinal vein was calculated for 1 minute at 2 disk diameters from the center of the optic disk. The total number of rolling platelets along all major veins was used as the number of rolling platelets in each rat. A platelet was defined as adherent to vascular endothelium if it remained stationary for >10 seconds. Adherent platelets were calculated as the total number of adherent platelets along all major retinal veins identified for 1 minute within a circle with a radius of 500 \(\mu\)m from the center of the optic disk. All parameters were evaluated after a stabilization period of 5 minutes after the administration of platelets.

### Semiquantification of P-Selectin and ICAM-I

#### Gene Expression

After 6 hours of reperfusion, 1 eye from each of 6 rats in the argatroban-treated, vehicle-treated, and nonoperated control groups was enucleated. Total RNA was isolated from the retina according to the acid guanidinium-thiocyanate-phenol-chloroform extraction method. \(^{29}\) The extracted RNA was quantified, and then 2 \(\mu\)g was used to make cDNA with use of a kit (OmniScript reverse transcriptase, QIAGEN). Polymerase chain reaction was performed with the method of Saiki et al., \(^{30}\) with slight modification. The following conditions were used: denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and polymerization at 72°C for 1 minute. The reaction was performed for 31 cycles. The primers were TGCTTG-GCTACTGGGACACTG (sense) and GGTTGCGACAGGACATGGTG (antisense) for P-selectin, AGCCTAGGCGCTTTAA (sense) and AGGGGTCCCAAGAGGTCTA (antisense) for ICAM-I, and GGCATCTGACCCCTGAAGTA (sense) and GC-CATGTCGTCGAGGTC (antisense) for \(\beta\)-actin. Nucleotide sequencing and restriction pattern analysis confirmed that polymerase chain reaction products were derived from the target cDNA sequences.
**Histologic Evaluation**

After 10 days of reperfusion, 1 eye each from 6 rats in the argatroban-treated, vehicle-treated, and nonoperated control groups was obtained to evaluate the severity of retinal damage. These eyes were fixed in 1.48% formaldehyde and 1% glutaraldehyde in phosphate buffer and then in 3.7% formaldehyde. The eyes were then dehydrated, embedded in paraffin, sectioned with a microtome at 4-μm thickness, and stained with hematoxylin and eosin. Each section was cut along the horizontal meridian of the eye through the optic nerve head; sections were cut perpendicular to the retinal surface. Retinal sections were examined with an optical microscope (×400) and then digitized by a charge-coupled device camera on a computer monitor.

To quantify retinal damage induced by transient ischemia, we measured changes in the thickness of the retina by using the method described by Hughes. Thickness of the inner plexiform layer and of the overall retina from outer to inner limiting membrane was measured.

**Statistical Analysis**

All values are mean±SEM. The data were analyzed by repeated-measure ANOVA, with post hoc comparisons tested with the Fisher protected least significant difference procedure. Differences were considered statistically significant when the probability values were <0.05.

**Results**

**PT and APTT**

Both PT and APTT were prolonged after reperfusion in the argatroban-treated group compared with the vehicle-treated group (Figure 1). APTT was significantly prolonged with treatment by argatroban at 4 (P=0.011) and 12 (P=0.0088) hours after reperfusion; PT was also significantly prolonged by treatment with argatroban at 4 (P=0.004) and 12 (P=0.036) hours after reperfusion.

**Physiologic Data**

The Table indicates the changes in physiologic variables and diameters of the major retinal vessels at various time points after transient ischemia. There were no significant differences between the argatroban-treated and vehicle-treated groups in any of the physiologic parameters studied.

**Leukocyte Rolling**

Immediately after AO was infused intravenously, only leukocytes were stained among the circulating blood cells. No rolling leukocytes were observed in the nonoperated control group. In the operated rats, some leukocytes were observed slowly rolling along major retinal veins but not along any major retinal arteries. In the vehicle-treated group, a few leukocytes were observed rolling along the venous walls at 4 hours after reperfusion. The flux of rolling leukocytes increased substantially and peaked at 12 hours after reperfusion (192.3±61.3 cells/min). In the argatroban-treated group, leukocyte rolling was significantly inhibited compared with that in the vehicle-treated group (P=0.015; Figure 2A). The number of rolling leukocytes in the argatroban-treated group was reduced to 9.9% of that in the vehicle-treated group at 12 hours after reperfusion (P=0.019).

**Leukocyte Accumulation**

Figure 2B indicates changes in the numbers of leukocytes accumulated in the retinal microcirculation in the argatroban-treated and vehicle-treated groups; few leukocytes could be found in the nonoperated control retinas. In the vehicle-treated group, accumulated leukocytes began to increase with time after reperfusion and peaked at 12 hours after reperfusion (934.6±196.0 cells/mm²). The number of accumulated leukocytes was significantly decreased in the argatroban-treated group compared with the vehicle-treated group (P=0.017). With argatroban treatment, the number of accumulated leukocytes was reduced to 41.3% at 12 hours after reperfusion (P=0.034).

**Platelet Rolling and Adhesion**

Immediately after labeled platelets were infused intravenously, fluorescent platelets were visibly circulating in the
retinal vessels. No rolling or adherent platelets were observed along the major retinal vessels in the nonoperated control rats. In the vehicle-treated group, some platelets were observed slowly rolling among many free-flowing platelets along major retinal veins but not along any major retinal arteries (Figure 3A). In the vehicle-treated group, some platelets were observed rolling along the venous walls 4 hours after reperfusion. The number of rolling platelets increased substantially and peaked at 12 hours (36.5±10.0 cells/min). Rolling platelets were inhibited significantly in the argatroban-treated group compared with the vehicle-treated group (P<0.0048; Figure 3B). The maximum number of rolling platelets at 12 hours was significantly reduced to 8.2% in the argatroban-treated group compared with the vehicle-treated group (P<0.0001).

P-Selectin and ICAM-1 Gene Expression

The levels of gene expression are shown as a ratio to the average values of nonoperated control rats (Figure 4). ICAM-1 mRNA expression was upregulated in the vehicle-treated group and was significantly suppressed in the argatroban-treated group at 6 hours after reperfusion (P=0.0048; Figure 3B). The maximum number of rolling platelets at 12 hours was significantly reduced to 8.2% in the argatroban-treated group compared with the vehicle-treated group (P=0.0078). In the vehicle-treated group, platelets adherent to the venous walls increased after reperfusion and peaked at 12 hours (12.7±1.2 cells/min). However, platelet adhesion was inhibited significantly in the argatroban-treated group compared with the vehicle-treated group (P=0.0023; Figure 3C). Moreover, the maximum number of adherent platelets at 12 hours was significantly reduced to 21.1% in the argatroban-treated group compared with the vehicle-treated group (P<0.0001).
P-selectin mRNA expression was also upregulated in the vehicle-treated group and was significantly suppressed in the argatroban-treated group \((P=0.0034)\). Histologic examination showed a decrease in retinal thickness of operated rats whether they were treated with argatroban or not. The decrease in retinal thickness was more severe in the inner than outer retina. Although retinal thickness was reduced in both groups, it was significantly better preserved in the argatroban-treated group than in the vehicle-treated group \((P=0.0002; \text{Figure 5})\). Furthermore, the protective effect was more substantial in the inner retina. The thickness of the inner plexiform layer in rats treated with argatroban was 183% of that in the vehicle-treated group \((P=0.0002)\).

**Discussion**

In the current study, histologic findings indicated a protective effect of argatroban against retinal injury after transient retinal ischemia. Our results demonstrated that argatroban could also suppress leukocyte–platelet–endothelial cell interactions in the postischemic rat retina. The expression of mRNA of P-selectin and ICAM-1 was suppressed significantly in the argatroban-treated retina. On the basis of these findings, we suggest that argatroban might attenuate neural injury after transient ischemia, not only by inhibiting coagulation but also by inhibiting inflammatory reactions mediated by accumulated leukocytes and platelets.

Argatroban is the only direct thrombin inhibitor that is now in use clinically. Other direct thrombin inhibitors, like hirudin and hirulog, are proteins, so their antigenicity makes it...
difficult to use them clinically. Heparin is a clinically used drug. However, to express its antithrombotic ability, heparin has to form a complex with antithrombin III. Moreover, because the effect of heparin is irreversible, the dose must be strictly individualized for each patient. In the current study, we used osmotic pumps to administer argatroban continuously for ≈24 hours after ischemia induction because the half-life of argatroban is ≈30 minutes. However, this short half-life allowed us to use this drug safely.

In our study, histologic examination demonstrated the protective effect of argatroban on ischemia-induced retinal damage. Many previous studies have shown that argatroban attenuates neuronal degeneration after forebrain transient ischemia.6,7 In those studies, cerebral blood flow recovered significantly better in the argatroban-treated group than in the vehicle-treated group. Most investigators believe that argatroban exerts its protective effects primarily through inhibition of coagulation after transient cerebral ischemia, resulting in improvement of cerebral blood flow and inhibition of vascular permeability.

Many reports have suggested that leukocytes play a major role in inflammatory injury after transient ischemia.8,9 When endothelial cells are activated by ischemia, adhesion molecules are expressed on the endothelial cells and lead to leukocyte–endothelial cell interaction through a multistep process.12 In this study, argatroban inhibited the mRNA expression of these adhesion molecules after transient retinal ischemia. The suppressed leukocyte–endothelial cell interaction in the postischemic retina of argatroban-treated rats was associated with the suppressed expressions of these adhesion molecules.

Platelets also interact with activated endothelial cells to accumulate in the area of injury.17,18 Thrombin activates endothelial cells and promotes the expression of P-selectin on them. In vivo studies have shown that platelets can roll on activated endothelium through P-selectin expressed on the endothelial cells17,18 and participate not only in coagulation but also in the inflammatory reaction in posts ischemic tissue. In addition, thrombin activates platelets and causes them to produce inflammatory mediators, such as serotonin, leukotrienes, thromboxane A2, monocyte chemotactic protein-3, and platelet-derived growth factor.20–22 Moreover, platelets recruit leukocytes to injured regions through the expression of adhesion molecules on their surfaces or via the production of cytokines.23,24 Suppressed platelet–endothelial cell interactions in the argatroban-treated group would thus contribute in part to the neuroprotective effects of argatroban.

In conclusion, we have demonstrated that argatroban can inhibit platelet–endothelial cell interactions and leukocyte–endothelial cell interactions in retinal tissue that has been injured by transient ischemia. These results suggest that argatroban attenuates ischemia/reperfusion injury not only by inhibiting coagulation but also by inhibiting inflammatory reactions mediated by leukocytes and platelets.

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References


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