Vascular Adhesion Protein-1 Plays an Important Role in Postischemic Inflammation and Neuropathology in Diabetic, Estrogen-Treated Ovariectomized Female Rats Subjected to Transient Forebrain Ischemia

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Received October 12, 2005; accepted December 6, 2005

ABSTRACT

Endothelial vascular adhesion protein-1 (VAP-1) facilitates leukocyte adhesion and infiltration. This relates partly to the function of VAP-1 as a semicarbazide-sensitive amine oxidase (SSAO). We examined the effects of VAP-1/SSAO inhibition [via LJP-1207 (\((N'(2-phenyl-allyl)-hydrazine hydrochloride)\)] on pial venular leukocyte adhesion and infiltration (at 2–10 h of reperfusion) and neuropathology (at 72 h of reperfusion) after transient forebrain ischemia (TFI). A model associated with increased postischemic inflammation was used—i.e., diabetic ovariectomized (OVX) female rats given chronic estrogen replacement therapy (ERT). We compared rats treated, either at the onset or at 6 h of reperfusion, with saline or LJP-1207. Additional rats, rendered neutropenic 24 h before TFI, were studied. In saline-treated controls, intravascular accumulation of adherent leukocytes gradually increased, reaching 15 to 20% of the venular area, at which point neutrophil infiltration commenced (at \(-6\) h). In the rats given LJP-1207 at the onset of reperfusion, limited neutrophil adhesion (\(<5\%\) maximum) and no infiltration were observed. These results generally paralleled those in neutropenic rats. In rats treated at 6 h of reperfusion, the pattern of neutrophil adhesion was similar to that of the saline-treated group up to 6 h, but further infiltration was essentially prevented. Neurologic outcomes and histopathology were similar to one another in the LJP-1207-treated and neutropenic groups and significantly improved over those in saline-treated controls. Thus, VAP-1-mediated post-TFI leukocyte infiltration provides a substantial measure of neuroprotection. This could explain the finding of LJP-1207 having at least a 6-h therapeutic window in this model.

Postischemic neuropathology has been linked to the increases in cerebral vascular leukocyte adhesion and infiltration that often occur after an ischemic event (reviewed by Frijns and Kappelle, 2002). Nevertheless, experimental evidence exists to challenge this view (reviewed by Emerich et al., 2002). Furthermore, recent clinical trials have failed to demonstrate any benefit from treatment with adhesion molecule antibodies or blockers (Sughrue et al., 2004). However, these clinical findings are confounded by factors related to immunogenicity (use of nonhuman antibodies) and inconsistencies in patient recruitment criteria.

A general implication one might derive from these mixed experimental findings is that, although increased leukocyte activity may accompany most cerebral ischemia episodes (especially those with reperfusion), it may not necessarily be a primary contributor to neuropathology in all cases. Results from our laboratory suggest that whether leukocytes contribute to ischemic neuropathology or not depends on whether leukocyte (principally neutrophil) (Arumugam et al., 2005) infiltration and not simply venular adhesion has occurred during the initial 10 to 12 h of reperfusion. Thus, the extent of neuropathology assessed after several days of reperfusion (Santizo et al., 2002) seemed to parallel the patterns of acute neutrophil extravasation (over 10 h of reperfusion), as viewed

ABBREVIATIONS: OVX, ovariectomized; VAP-1, vascular adhesion protein-1; SSAO, semicarbazide-sensitive amine oxidase; TFI, transient forebrain ischemia; LJP-1207, \((N'(2-phenyl-allyl)-hydrazine hydrochloride)\); STZ, streptozotocin; E\(_2\), 17\(\beta\)-estradiol; CBF, cerebral blood flow; PMNL, polymorphonuclear leukocyte; MPO, myeloperoxidase; FJ, Fluoro-Jade B; MAO, monoamine oxidase; COX, cyclooxygenase.
in pial venules in situ (Xu et al., 2004). It is relevant to note that these experiments were performed on diabetic, intact, ovariectomized (OVX), and estrogen-treated OVX females to test the hypothesis that chronic estrogen replacement therapy is associated with a potentiation of postischemic inflammation and neuropathology, opposite to what was previously observed in nondiabetic females (Santizo et al., 2000b). These observations may be of particular interest to postmenopausal females, because women in this age group not only are at greater risk for cerebral ischemic events, but they also often experience an increased incidence of reduced glucose tolerance and hyperglycemia, although not necessarily related to estrogen depletion per se. Furthermore, recent clinical findings failed to demonstrate any cardiovascular or brain-protective effects of chronic estrogen replacement therapy in postmenopausal women, contrary to results obtained in experimental animals. Therefore, studies using clinically relevant experimental models associated with a loss of estrogen's neuro- and vasculoprotective effects are important, insofar as they may yield clues that can be used in devising estrogen replacement strategies that will be beneficial.

In the present investigation, we sought to establish a link between the increased postischemic leukocyte infiltration and neuropathology we observed in our earlier studies in diabetic OVX females given chronic estrogen replacement therapy. To that end, we targeted vascular adhesion protein-1 (VAP-1). This protein was identified as playing an important role in promoting leukocyte adhesion as well as transmigration. Those actions of VAP-1 may relate to its direct function as an adhesion, in addition to its enzymatic function as a semicarbazide-sensitive amine oxidase (SSAO) (Salmi et al., 1993; Stolen et al., 2005). In addition to a soluble form found in the blood, VAP-1/SSAO is concentrated in vascular tissue, including cerebral microvessels and endothelial cells (Salmi et al., 1993) and is mobilized to the luminal surface of endothelial cells in the face of an inflammatory stimulus. However, no studies have addressed whether VAP-1/SSAO influences postischemic leukocyte trafficking in the brain. In this study, using diabetic, estrogen-treated OVX females subjected to transient forebrain ischemia (TFI), we examined the role of VAP-1/SSAO in 1) neutrophil adhesion and infiltration during the initial 10 h of reperfusion and 2) the level of neuropathology at 72 h of reperfusion. Thus, rats were studied in the absence and presence of a novel, highly selective VAP-1/SSAO inhibitor, LJP-1207 [N’-(2-phenyl-al-lyl)-hydrazine hydrochloride] (Salter-Cid et al., 2005). To confirm the importance of neutrophils, rats rendered neutropenic 24 h before ischemia were also studied. It was found that, when the VAP-1/SSAO inhibitor was given at the onset of reperfusion, similar to findings in neutropenic rats, neutrophil adhesion was substantially reduced and infiltration, which normally appears after ~6 h postischemia in these rats, was not observed. When the inhibitor treatment was delayed until 6 h of reperfusion, infiltration, but not intravascular adhesion, was markedly attenuated relative to saline-treated controls. Irrespective of treatment timing, significant neuroprotection was seen in the presence of the VAP-1/SSAO blocker as well as in neutropenic rats.

**Materials and Methods**

**Animals.** The study protocol was approved by the Institutional Animal Care and Use Committee. OVX, female Sprague-Dawley rats (200–250 g at arrival) were used. Ovariectomies were performed by the vendor (Charles River, Wilmington, MA) 1 week before shipment. Diabetes was induced via streptozotocin (STZ) (60 mg/kg i.v.) at ~1 week after arrival, and the rats were studied ~6 to 8 weeks later. At 1 week before the study, 17β-estradiol (E2) treatment was initiated (0.1 mg/kg/day i.p. for 7 days). Previous work from our laboratory demonstrated that the 0.1-mg/kg daily dose of E2 resulted in an average daily plasma E2 concentration that falls between the peak and nadir levels observed over the normal rat estrous cycle (Xu et al., 2004).

**Ischemia Model.** Right forebrain ischemia was produced by clamping the right common carotid artery, combined with blood withdrawal from the subclavian vein, to decrease cortical blood flow to 20% of baseline, as measured by laser Doppler flowmetry. Reperfusion was established after 20 min via removal of the carotid clamp and reinfusion of withdrawn blood over a 15-min period. The exact procedure used, with respect to anesthesia, muscle relaxation, and surgical preparation, depended upon whether the animals were to be used in leukocyte adhesion/infiltration evaluations or long-term recovery and neuropathologic assessments (see below). Results from pilot and published (Wang et al., 1999) investigations established that intraischemic CBF relationships between the cortex and subcortical structures did not vary with hormone or glycemic status. Furthermore, cortical CBF recovered to preischemic levels within 2 min after onset of reperfusion and remained within 80% of that level for as long as monitoring continued (up to 4 h). In preliminary evaluations, we did not observe any differences in postischemic CBF recovery when comparing selected rats from each group (see below).

**Experimental Groups.** The studies were organized into two major experimental series. In the first series, pial venular leukocyte adhesion was monitored over the initial 10-h reperfusion period. In the second series, postischemic neuropathology was assessed in rats exposed to 72 h of reperfusion. For leukocyte adhesion evaluations, rats were prepared with closed cranial windows 24 h before study. The procedure for “chronic” placement of cranial windows was described in a previous article from our laboratory (Xu et al., 2001). Each series was further divided into four subgroups. Subgroup 1 received 30 mg/kg i.v. of the VAP-1/SSAO inhibitor LJP-1207 at the onset of reperfusion. In subgroup 2, LJP-1207 treatment was given at 6 h of reperfusion. The third subgroup was a vehicle (saline)-injected control. Subgroup 4 rats were rendered neutropenic before ischemia. For these experiments, rats were injected i.v. with 0.3 ml of a rabbit anti-rat PMNL antibody (diluted 1:1; Research Diagnostics, Inc., Flanders, NJ) 24 h before ischemia. In some control and neutropenic rats (three to four each), arterial blood samples were obtained before ischemia, and leukocyte composition was examined using hemocytometry.

**Leukocyte Adhesion/Infiltration Assessments.** In series 1 experiments, on the day of study, the rats were anesthetized with halothane. Paralysis was then induced with curare, followed by tracheotomy and mechanical ventilation. During surgery, anesthesia was maintained with 1.2% isoflurane-70% N2O-30% O2. The femoral arteries and veins were cannulated for blood sampling, arterial pressure monitoring, and drug infusions. Rectal temperature was servo-controlled at 37°C with a heating pad. The right common carotid artery was isolated, followed by insertion of a right subclavian venous catheter. The previously implanted cranial window was then re-exposed. After the surgery, the isoflurane was discontinued, and the rat was maintained on 70% N2O-30% O2-fentanyl (25 μg/kg/h i.v.) thereafter. The space under the window was filled with artificial cerebrospinal fluid that was equilibrated with 10% O2-5% CO2 with balance of N2. The 37°C artificial cerebrospinal fluid was infused at 0.5 ml/min. A 0.8-mm-diameter laser Doppler flow probe (Perimed, Jarfalla, Sweden) was secured to the cranial window above the right parietal cortex and baseline measurements, in perfusion units, were recorded. The leukocyte activity of pial venules was monitored using a rhodamine-filtered digital video camera (Photometrics; Fryer Co.
Inc., Huntley, IL), attached to a microscope. Leukocytes were labeled with rhodamine 6G (200 μg/ml in 0.9% saline). The rhodamine was given initially as an i.v. bolus (1 ml) followed by a continuous infusion at a rate of 1 ml/h (Santizo et al., 2000b). Images of leukocytes were captured and saved, using the MetaMorph software system (Universal Imaging Corporation, Downingtown, PA), and displayed on a computer monitor. Leukocyte dynamics were monitored after 2, 4, 6, 8, and 10 h of reperfusion. Illumination was limited to <60 s at a time to avoid phototoxicity. Leukocyte adherence was measured in all experiments as the area percentage of leukocytes overlapping the viewed venular area. The viewed venular area was determined from frames captured following switching from fluorescence to standard light microscopy. The value of the viewed venular area was expressed as a percentage of the total area exposed in the captured frame. Expressing leukocyte adherence (and infiltration—see below) as a fractional area, rather than the number of rhodamine-positive cells per unit venular area was necessitated by the two-dimensional view generated by our video/microscopy system. Thus, we could not readily distinguish overlapping firmly adherent (or extravasated) cells from one another. Such overlap became increasingly prominent with time in a number of instances. In many of the saline-treated controls (i.e., non-LJP-1207-treated; non-neuropenic), leukocyte infiltration was seen (but only at time points >6 h). When extravasation was observed, another measurement strategy was applied. Thus, in addition to the measurement of leukocyte adherence described above, the total area of rhodamine-positive cells was counted, including cells that were distinctly extravascular. In both instances, the denominator was viewed venular area. An additional calculation was then made, in which the percentage of “adherent” leukocytes was subtracted from the total leukocyte percentage. The result provided us with an “index of infiltration”. It should be noted that in rats from the above diabetic groups not rendered ischemic, but where pial venular leukocytes were monitored over 10 to 12 h (sham ischemic time controls; data not shown), the level of leukocyte adhesion remained low throughout the monitoring period. In some of the rats from each group, at the termination of the study, brains were harvested for immunohistochemical analysis of myeloperoxidase (MPO) expression and histopathologic analysis via Fluoro-Jade (see below). This was done primarily to evaluate whether leukocyte (neutrophil) expression and histopathologic analysis via Fluoro-Jade (see below). brains were subsequently removed and processed for histologic examination. Analyses of LJP-1207 Selectivity, Efficacy, and Pharmacokinetics. LJP-1207 (formula weight of 184.6), which, by virtue of a hydrazine functional group, is a potent, selective inhibitor of VAP-1/SSAO enzyme activity. That selectivity was established in pilot studies, in which LJP-1207 was screened for potential cross-reactivity with a variety of enzymes. Rat VAP-1/SSAO activity was measured in rat lung homogenates using a radiochemical assay (Lizzano et al., 1998). Briefly, the tissue was homogenized 1:20 (w/v) in 10 mM phosphate-buffered saline and centrifuged at 1000g for 10 min at 4°C. VAP-1/SSAO activity in 100 μl of the supernatant was determined using 20 μM [14C]benzylamine as substrate and, when appropriate, added LJP-1207. The reaction was carried out at 37°C in a final volume of 400 μl of 100 mM potassium phosphate buffer (pH 7.2) and stopped with 100 μl of 2 M citric acid. Radioactively labeled products were extracted into toluene-ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole before liquid scintillation counting. All experiments were performed in the presence of 1 μM dorglyline and pargyline to inhibit monoamine oxidase (MAO)-A and MAO-B activities, respectively. Inhibition was calculated as percent inhibition compared with saline-treated control after correcting for background absorbance, and IC50 values were calculated using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). MAO-A and MAO-B activities were measured using a coupled colorimetric method (Holt et al., 1997). Recombinant human MAO-A and human MAO-B enzymes were obtained from BD Biosciences (Bedford, MA). The assay was performed in 96-well microtiter plates as follows. A predetermined amount of inhibitor diluted in 0.2 M potassium phosphate buffer, pH 7.6, was added to each well of a 96-well microtiter plate. The final concentration of inhibitor was between 50 nM and 1 mM. Controls lacked inhibitor. The following agents were then added to a final reaction volume of 200 μl in 0.2 M potassium phosphate buffer, pH 7.6, 0.04 mg/ml of MAO-A or 0.07 mg/ml MAO-B enzyme, 15 μl of 10 mM tyramine substrate (for MAO-B), and 50 μl of freshly made chromogenic solution containing 750 mM vanillic acid (Sigma), 400 nM 4-aminooxanthine (Sigma, St. Louis,
MO), and 12 U/ml horseradish peroxidase (Sigma). The plates were incubated for 1 h at 37°C, and the increase in absorbance, reflecting MAO activity, was measured at 490 nm using a microplate spectrophotometer (Power Wave 40; Bio-Tek Instruments, Winooski, VT). Clorgyline (0.5 μM) and pargyline (10 μM), inhibitors of MAO-A and -B, respectively, were added to some wells as positive controls for MAO inhibition. The level of inhibition was calculated as percent inhibition compared with control after correcting for background absorbance, and IC50 values were calculated using GraphPad Prism software. The inhibitory effects of LJP-1207 toward cyclooxygenase (COX)-1, COX-2, matrix metalloproteinase-9, protein kinase C, caspase 3, cathepsin G, and xanthine oxidase were assayed by MDS Pharma Services (Taipei, Taiwan). The methodologic references can be found at http://discovery.mdsps.com/Catalog/. With the exception of COX-1 (IC50 = 3 μM), the IC50 values of LJP-1207 toward the remaining enzymes exceeded 10 μM. In separate assays, we evaluated the level of VAP-1/SSAO inhibition at 6 h after i.v. administration of LJP-1207 or saline in nonischemic, diabetic, E2-treated OVX females (n = 6, each group). Samples of lung and pial tissue were obtained from each animal. Enzyme activities in lung tissue samples were measured in each animal, whereas the pial samples were pooled, according to treatment group, before the biochemical assay (Lizcano et al., 1998). For this assay, 100 μl of sample (diluted 1:20, w/v, in phosphate-buffered saline) were used in each reaction. The backgrounds were determined by preinhibition of each sample with 1 mM semicarbazine, 1 μM pargyline, and 1 μM clorgyline for 30 min.

Plasma pharmacokinetics of LJP-1207 were determined in three rats by a contract research service company (Cerep, Redmond, WA). LJP-1207 (1 mg/kg) was injected intravenously. Blood was obtained from a jugular vein catheter at 5, 15, 60, 120, 240, 360, and 1440 min. Aliquots were collected in tubes coated with lithium heparin, and plasma was harvested and kept frozen at −20°C until analysis. Samples were processed using acetonitrile precipitation and analyzed by high-performance liquid chromatography-tandem mass spectrometry. The data were used to generate concentration versus time curves and allow the determination of fundamental pharmacokinetic data (e.g., Cmax, Tmax, area under the curve, clearance, terminal elimination half-life, oral bioavailability, and volume of distribution) using WinNonlin software (Pharsight, Mountain View, CA).

Statistics. For parametric data, statistical analyses were performed using a paired t test and one-way analysis of variance with a Bonferroni post-test and one-way analysis of variance for non-parametric data (neuromorphic outcomes), we used a one-way analysis of variance on ranks, with post hoc analysis via Dunn’s method. A level of p < 0.05 was considered significant in all statistical tests. Values are presented as means ± S.D. All drugs/chemicals were obtained from Sigma, unless otherwise stated.

Results

LJP-1207 Pharmacology. The IC50 values of LJP-1207 toward VAP-1/SSAO, MAO-A, and MAO-B, measured in purified enzyme or homogenized tissue preparations, are provided in Table 1. Thus, LJP-1207 displayed a 103 to 104 times greater potency toward VAP-1/SSAO compared with the other amine oxidases. The choice of the 30-mg/kg dose of LJP-1207 used in the present study was guided by evaluation of its plasma pharmacokinetics, the selectivity data provided in Table 1 (as well as the data provided by MDS Pharma Services; see Materials and Methods), and measurements of SSSAO activity in lung and pial tissue at 6 h after i.v. injection. The elimination half-life of LJP 1207 after a 1-mg/kg i.v. administration was 183 min with a clearance of 97 ml/min/kg (n = 3). The maximum concentration observed at the first time point, 5 min after injection, was 292 ng/ml. A linear extrapolation of the 1-mg/kg pharmacokinetic data results in an estimated peak plasma level of LJP-1207 of approximately 30 μM at the 30-mg/kg dose. At 6 h after LJP-1207 administration, the relative levels of VAP-1/SSAO activity in lung tissue harvested from E2-treated OVX females were significantly decreased to 31 ± 4% of the level measured in saline-injected rats (n = 6). In the pooled pial samples, VAP-1/SSAO activity in the LJP-1207-treated rats was 40% lower than that seen in the saline-treated animals. These data indicate that although there is substantial exposure to LJP-1207 at the doses used in this study, the concentrations of LJP-1207 achieved in the circulation, at 6 h post-treatment, remain well within the drug’s selectivity range. The apparently lower magnitude reduction in VAP-1/SSAO activity in pial versus lung tissue may reflect some blood-brain barrier impediment to drug passage into cerebral vascular cells. However, because the pial tissue used in these assays was not obtained from rats exposed to ischemia and reperfusion, it is likely that the level of VAP-1/SSAO inhibition measured in these samples is of a lesser magnitude than one might observe in the postischemic reperfusion period. That is, some diminished blood-brain barrier function can be anticipated postischemically, thereby allowing passage of more drug into the brain and cerebral vascular cells.

Arterial Blood Variables. Arterial partial CO2 pressure and partial O2 pressure measurements obtained before 20 min of ischemia, at the end of ischemia, and during 10 h of reperfusion (series 1) remained between 32 and 38 mm Hg and >100 mm Hg, respectively, during the entire study. As commonly seen with this model (Xu et al., 2004), end-ischemic arterial pH was significantly lower than the preischemic value in all experimental groups (mean ± S.D. end-ischemic versus preischemic values for all groups were 7.39 ± 0.02 versus 7.48 ± 0.02, respectively). Preischemic mean arterial blood pressure values were similar among groups and were reduced, during ischemia, to 28 to 32 mm Hg. Plasma glucose values, in samples obtained immediately before ischemic onset, were 27.6 ± 1.4, 27.2 ± 2.0, 26.3 ± 2.8, and 28.1 ± 4.2 mM in the saline-treated, LJP-1207-0, LJP-1207-6, and neutropenic groups, respectively. In rats subjected to 20 min of ischemia and 72 h of reperfusion (series 2), peri-ischemic arterial partial CO2 pressure, partial O2 pressure, pH, and mean arterial blood pressure showed variations similar to those seen in series 1 experiments, and plasma glucose values were 27.0 ± 3.7, 26.0 ± 7.4, 30.3 ± 5.1, and 27.8 ± 6.5 mM in the saline-treated, LJP-1207-0, LJP-1207-6, and neutropenic groups, respectively.

Postischemic Leukocyte Adhesion. In saline-treated control E2-treated diabetic OVX females, leukocyte adhesion showed a progressive increase between 2 and 6 h of reperfusion (Fig. 1A). The majority of these rats (four of seven) displayed an extravascular presence of leukocytes beginning at around the 6-h time point of reperfusion (Fig. 2). By 10 h of reperfusion, six of the seven rats in the saline-treated
group exhibited extravasated leukocytes. Quantitatively, that is reflected by higher values for total leukocyte presence (Fig. 1B) versus leukocytes in contact with cerebral venules (Fig. 1A). This is illustrated in Fig. 1C, where the calculated differences between total and adherent leukocytes are summarized. Only the 6- to 10-h reperfusion values for the saline- and the 6-h treatment groups are displayed in Fig. 1C, because only rats from those groups exhibited any detectable extravascular leukocyte presence during that time period. On the other hand, in rats treated with the VAP-1/SSAO inhibitor, LJP-1207, at the onset of reperfusion (0 h), leukocyte adhesion remained significantly below the levels observed in the saline- and the 6-h treatment groups throughout the 10-h reperfusion period. Furthermore, no signs of extravasation were seen in this group (Fig. 2), as reflected in identical values when leukocyte adhesion (Fig. 1A) was compared with total leukocyte presence (Fig. 1B). In rats in which LJP-1207 treatment was given at 6 h of reperfusion, the temporal pattern of postischemic leukocyte adhesion tracked that of the saline-treated control group out to 6 h (Figs. 1A and 2). In addition, in three of the eight rats studied in the 6-h treatment group, a slight degree of leukocyte infiltration was seen...
at 6 h of reperfusion and thereafter, although comparisons of total leukocyte presence (Fig. 1B) and leukocyte adhesion (Fig. 1A), at given time points over the 6- to 10-h time frame, yielded no statistically significant differences. Treatment of the rats with the anti-PMNL antibody was found to be effective in selectively depleting circulating neutrophils. Thus, antibody treatment reduced blood neutrophil content to barely detectable levels (from 4176 ± 1796 to 63 ± 210 neutrophils/mm³), without having any significant effect on lymphocyte content (10,419 ± 3892 versus 7043 ± 4761 lymphocytes/mm³ in vehicle versus antibody-treated rats, respectively). In the neutropenic rats, far fewer adherent cells were observed over the 10-h period of reperfusion, to the extent that a <3% pial venular occupancy was observed at 6 and 10 h (Fig. 1, A and B; p < 0.05 versus saline-treated control and LJP-1207-6, at all time points). Comparisons between the neutropenic and LJP-1207-0 groups revealed no statistically significant differences. These findings not only support the effectiveness of the anti-PMNL strategy but also indicate that PMNLs comprise a substantial fraction of the entire mass of adherent blood cells seen during the initial hours of ischemic reperfusion.

Examination of coronal sections prepared from brains harvested at 10 to 12 h of reperfusion revealed the presence of extravascular PMNLs (MPO immunoreactivity) in the ischemic hemispheres of saline-treated rats, especially in the cerebral cortex. This was observed both at deep and superficial layers of the cortex (Fig. 3). In the LJP-1207-6 group, MPO expression was detected almost exclusively in association with blood vessels (Fig. 3). In the LJP-1207-0 and neutropenic (not shown) groups, little or no MPO expression was observed. Thus, at least in the cortex, these findings seem to track what was observed in the intravital microscopy evaluations. However, a rather limited intravascular MPO immunoreactivity was seen in the ischemic striatum, hippocampus, and thalamus (Fig. 3; results for hippocampus not shown)—regions that displayed areas of neuronal damage in saline-treated animals (see Histopathology).

**Neurologic Outcomes.** The neurologic outcome scores (3-day total) for the four groups are summarized in Fig. 4. The nonparametric data are presented as a scatterplot, with median values for each group. Because no significant differences were observed when comparing saline-treated rats run in parallel with the two LJP-1207-treated and the anti-PMNL-treated groups, the data from all control groups were combined. A significantly greater neurologic impairment was noted when the control group was compared with the LJP-1207-0, LJP-1207-6, and neutropenic groups (median scores of 19, 2, 4, and 4, respectively).

**Histopathology.** As a general observation, FJ staining of brain sections in saline-treated control rats revealed damage in the medial thalamus and scattered throughout the cortex, in addition to the hippocampus and dorsolateral striatum. These results are consistent with published information derived from studies on hyperglycemic rats subjected to TFI (Li et al., 1998a,b; He et al., 2003; Ding et al., 2004). Far fewer numbers of degenerating (i.e., FJ-positive) cells were observed in the LJP-1207-treated and neutropenic groups (Fig. 5). In several rats from each group, brains were obtained for

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**Fig. 3.** MPO immunohistochemistry (at 10–12 h of reperfusion), depicting neutrophil presence in the cerebral cortex (A–E) of saline-treated (A–C), 0-h LJP-1207-treated (D), and 6-h LJP-1207-treated (E) rats and in the striatum (F) and thalamus (G) of saline-treated controls. Note the presence of extravascular neutrophils in the cortex of the controls, whereas in the 0- and 6-h LJP-1207-treated rats, MPO immunoreactivity remained associated with blood vessels. Note also the limited neutrophil presence in the ischemic striatum and thalamus. Scale bars are 100 μm (A) or 50 μm (B–G).
Fluoro-Jade analysis after the 10- to 12-h period of leukocyte monitoring. No FJ reactivity was detected in any of these animals (not shown). Thus, neuronal damage appears to occur well after the onset of neutrophil extravasation, making it highly unlikely that neutrophils played any sort of “bystander” role (Emrich et al., 2002). Examples of the patterns of brain cell damage seen in the saline- and the two LJP-1207-treated groups are presented in Figs. 6 and 7. The neutropenic group is not represented because these rats exhibited relatively few FJ-positive cells (similar to the rats treated with LJP-1207 at the onset of reperfusion). The pattern seen in the saline-treated group is similar to that reported in an earlier publication from our laboratory (Santizo et al., 2002). It should be mentioned that almost no FJ-positive cells were observed in the nonischemic left hemisphere in any animal. These figures also include higher magnification views in the cortex, striatum, and thalamus. The higher magnification view revealed that the FJ-positive cells had the appearance of neurons. In saline-treated controls, the greatest numbers of degenerating neurons were seen in the cortex and striatum (Fig. 6, A and D). In fact, all 10 control rats evaluated showed damage in these two regions. The presence of neurodegeneration in the thalamus was detected in 80% of the saline-treated controls (Fig. 7A), whereas hippocampal damage was seen in 70% of the controls (Fig. 7D). In the hippocampus, hilar (CA4) damage was the most frequently observed pathologic change. FJ-positive CA4 pyramidal cells were sometimes accompanied by the presence of FJ-reactive cells in the CA1 (not shown) and dentate regions. In contrast, in the rats treated with the VAP-1/SSAO inhibitor at the onset of reperfusion, no hippocampal damage was observed (Fig. 7E), and only a few isolated degenerating neurons were observed in the cortex (Fig. 6B), striatum (Fig. 6E), and thalamus (Fig. 7B). In rats treated with LJP-1207 at 6 h of reperfusion, significantly fewer FJ-positive neurons were observed in the above regions relative to saline-treated controls. When the two treated groups were compared, a significantly greater number of FJ-positive cells were seen in the cortex of the 6-h treatment group (Figs. 5 and 6C), and two of the six 6-h rats (Fig. 7F) showed degenerating neurons in the CA4 (but none in the CA1 or dentate region). To provide a measure of validation for the FJ results, we compared adjacent coronal sections, one of which was FJ-stained and the other was stained with H&E. We found very good agreement in representative brain section comparisons. This was seen in cortical, striatal, hippocampal, and thalamic views. Three examples are provided in Fig. 8. The first is taken at the level of the dentate/hilar region of the hippocampus in a saline-treated rat (Fig. 8, A and B). The second is taken from the same region in a 6-h LJP-1207-treated (Fig. 8, C and D) rat. The third example is from the cerebral cortex of a saline-treated control rat (Fig. 8, E and F). In Fig. 8, A to D, one should particularly note the distinction between the dorsal and ventral aspects of the dentate region. That is, the pyknotic-appearing granule cells of the ventral aspect (Fig. 8B) display substantial FJ reactivity (Fig. 8A), as opposed to the normal-appearing granule cells of the dorsal dentate, which show little or no FJ reactivity. In contrast, in the H&E-stained section obtained from the 6-h LJP-1207-treated rats, the dentate granule cells appear to be undamaged (Fig. 8D). No FJ reactivity was observed in those cells (Fig. 8C). Moreover, the transition from normal appearing to pyknotic hilar neurons seen in the H&E-stained sections (Fig. 8, B and D) was reflected in the pattern of FJ staining (Fig. 8, A and C).

**Discussion**

There were several key findings in this study. First, in agreement with a recent report from our laboratory (Xu et al., 2004), chronic E4 replacement therapy in diabetic O VX female rats was associated with substantial time-dependent increases in cerebral leukocyte adhesion, over the initial 6 h of reperfusion after forebrain ischemia. Subsequently, neutrophil infiltration was observed. Second, inhibition of VAP-1/SSAO at the onset of reperfusion, as well as preischemic neutrophil depletion, resulted in marked reductions in leukocyte adhesion and an absence of neutrophil infiltration.
Third, delaying inhibitor treatment until 6 h of reperfusion prevented infiltration without affecting the initial (i.e., 0–6 h) pattern of neutrophil adhesion. Fourth, both VAP-1/SSAO inhibition protocols and neutropenia were associated with significantly less neuropathology compared with that in saline-treated controls. Thus, with respect to interventions that restrict postischemic neutrophil infiltration in E2-treated diabetic OVX female rats subjected to forebrain ischemia, a therapeutic window of at least 6 h appears to be present.

We used Fluoro-Jade staining to identify histopathology. Previous studies have shown excellent agreement when comparing Fluoro-Jade to other accepted techniques for identifying neuronal damage or loss in the brain in neuropathology models (Kubova et al., 2001; Wang et al., 2003; Anderson et al., 2005; Bendel et al., 2005; Siman et al., 2005) (see also Fig. 8). One advantage of Fluoro-Jade is that damage is more readily detected compared with other commonly employed techniques. This is exemplified in direct comparisons between sections prepared with Fluoro-Jade and H&E-stained sections (Fig. 8). A potential drawback with Fluoro-Jade is that, under some circumstances, non-neural cells, such as astrocytes, may become Fluoro-Jade-positive. However, evidence indicates that such “extraneuronal” staining may only be observed after prolonged recovery periods (i.e., weeks, rather than days) (Butler et al., 2002). In the present study, in which 3-day recovery periods were used, higher magnification views of brain regions displaying Fluoro-Jade reactivity revealed cells with neuronal morphology.

Fig. 6. Representative photomicrographs showing Fluoro-Jade-positive neurons in the cortex (A–C) and striatum (D–F) in saline-treated control rats (A and D) and in rats treated with LJP-1207 at the onset (LJP-1207-0h; B and E) or at 6 h (LJP-1207-6h; C and F) of reperfusion. Horizontal bar, 100 μm. Inset depicts higher magnification view of the area outlined.

Fig. 7. Representative photomicrographs showing Fluoro-Jade-positive neurons in the thalamus (A–C) and in the CA4 (hilar) region of the hippocampus (D–F) in saline-treated control rats (A and D) and in rats treated at the onset (LJP-1207-0h; B and E) or at 6 h (LJP-1207-6h; C and F) of reperfusion. Horizontal bar, 100 μm. Inset depicts higher magnification view of the area outlined.
The influence of VAP-1/SSAO on the adhesion-transmigration cascade may occur at multiple sites. Thus, results comparing wild-type and VAP-1/SSAO knockout mice (Stolen et al., 2005), using an in vivo model of cytokine-induced cremaster inflammation, indicated that VAP-1/SSAO may play independent roles in supporting both firm adhesion of leukocytes and leukocyte transmigration. Such a dual role for VAP-1/SSAO could account for the present findings, in which inhibitor treatments applied around the onset of leukocyte infiltration (6-h reperfusion) (Xu et al., 2004) were associated primarily with preventing diapedesis, whereas vascular adhesion of leukocytes remained at the higher levels seen in the absence of treatment. That is, a restriction of vascular adhesion was not required for LJP-1207 to prevent neutrophil infiltration.

Findings in rodents subjected to transient focal ischemia point to a temporally progressive adhesion and infiltration of leukocytes following restitution of flow, with neutrophils leading the way. Moreover, in many instances, anti-leukocyte strategies were effective in reducing neuropathology (e.g., Frijns and Kappelle, 2002). A limited number of published reports have examined postischemic leukocyte contributions in forebrain/global ischemia models. Analysis of results from those studies in normoglycemic males is complicated by the fact that a number of different models were used, including several large vessel occlusion models (Dinnagl et al., 1994; Beck et al., 1997; Hudetz et al., 1999; Abels et al., 2000) and cardiac arrest (Bottiger et al., 1998). With the exception of cardiac arrest, postischemic leukocyte adhesion was rather modest. In the rats subjected to cardiac arrest and recovery, a significant elevation in MPO immunoreactive cells (principally neutrophils) were observed in the cortex at 6 h and 1 week of recovery. However, the presence of neutrophils did not correlate well with the injury patterns in other brain regions. In nondiabetic female rats, we found an inverse relationship between estrogen and postischemic leukocyte adhesion (Santizo et al., 2000b), at least out to 6 h of reperfusion. In the 6- to 10-h reperfusion interval (H. L. Xu and D. A. Pelligrino, unpublished data), only untreated OVX females, but not intact and E2-treated OVX females, exhibited signs of infiltration. On the other hand, in the presence of hyperglycemia, present and published (Lin et al., 2000; Xu et al., 2004) findings indicate an exacerbation of leukocyte trafficking following transient forebrain ischemia. Furthermore, these hyperglycemic animals displayed greater levels of neuropathology (see also Santizo et al., 2002) compared with animals in which postischemic leukocyte activity was modest.

However, such findings can only be considered as correlative. Indeed, one of the key criticisms raised in opposing a pathogenic role for neutrophils in ischemia/reperfusion models is the absence of evidence showing significant neutrophil trafficking in advance of neuronal damage (Emerich et al., 2002). In fact, in one publication (Ahmed et al., 2000), it was reported that increasing peri-ischemic leukocyte activity, via lipopolysaccharide pretreatment 24 h before temporary middle cerebral artery occlusion, was associated with reduced brain damage. However, lipopolysaccharide pretreatment induces tolerance to ischemia through a variety of mechanisms (Kariko et al., 2004). Thus, the impact of the findings by Ahmed et al. (2000), as they apply to the role of leukocytes in ischemic brain damage, is questionable. In the present study, increased neutrophil trafficking in advance of brain damage was seen. Moreover, the various interventions designed to specifically impede that process, but at different steps (i.e., neutropenia or early and delayed VAP-1/SSAO inhibitor administration), were all efficacious in restricting neutrophil infiltration and were all associated with reduced neuropathology. This is compelling evidence favoring a causative role for neutrophils in the postischemic neuronal damage seen in diabetic OVX rats given chronic estrogen replacement. However, because lymphocytes represent the major white blood cell type in rats and lymphocyte trafficking is regulated by VAP-1/SSAO (Salmi et al., 1993), possible contributions from lymphocytes warrant consideration. Limited evidence to date (focal ischemia/reperfusion only) indicates that lymphocyte...
infiltration occurs much later (days), compared with neutrophils (Arumugam et al., 2005). If a similar time differential in forebrain ischemia/reperfusion is assumed, it appears unlikely that the effects of LJP-1207 observed in the present study relate in any meaningful way to actions toward lymphocytes. One reason is that the half-life for the drug in rats is only 3 h. It is improbable that sufficient LJP-1207 remains in the circulation 2 to 3 days after its administration. Another finding favoring a role for neutrophils over lymphocytes relates to the capacity for the anti-PMNL antibody to impair neuroprotection in the absence of any significant effects on lymphocyte numbers.

The processes used by neutrophils to mediate brain damage can include release of reactive O2 species and proteases. Both, but especially the latter, when released into the brain parenchyma can lead to damage of the extracellular matrix and blood-brain barrier (Gidday et al., 2005) and facilitate the appearance of the so-called "hemorrhagic transformation" (Wang et al., 2004).

One confounding observation in the present study relates to the presence of extravascular PMNLs in brain regions exhibiting neuropathology. Thus, the degree of leukocyte infiltration observed after 10 h of reperfusion when pial venules were viewed was mirrored throughout the ischemic cerebral cortex—a region displaying substantial neuronal damage in saline-treated controls. Curiously, in the deeper forebrain structures that typically display postischemic neuropathology in association with diabetes and hyperglycemia (i.e., hippocampus, striatum, and thalamus), only a modest presence of PMNLs was observed in brains harvested at 10 to 12 h of reperfusion. These latter observations do not necessarily obviate contributions from leukocytes to the damage seen in these deeper structures. Because information regarding the 12- to 72-h period of reperfusion is lacking, the possibility remains that leukocyte activity may peak later in those regions in relation to the cortex (see, for example, Bottiger et al., 1998; Lin et al., 2000). Another possibility that merits consideration is that the cortex may represent the proximal site of neuronal damage. This could result in loss of cortical afferent inputs to those other structures and contribute to the subsequent appearance of neuropathology (e.g., Block et al., 2005). Thus, the link between PMNL trafficking and damage to the other structures might only be indirect. This could explain why anti-PMNL strategies, in the present study, protected both the cortex and the deeper structures as well, despite limited leukocyte activity in the subcortical regions.

In conclusion, chronic E2 replacement in diabetic OVX females is associated with a pronounced exacerbation of leukocyte adhesion and infiltration following transient forebrain ischemia. The opposite effect is seen in nondiabetic females. Those opposing actions of chronic E2 replacement on leukocytes are paralleled by neuropathologic findings. A similar result, with respect to E2 replacement potentiating postischemic leukocyte activity and brain damage in diabetic OVX females, was recently reported for rats subjected to temporary middle cerebral artery occlusion (Yong et al., 2005). The results of the current study also indicated that VAP-1/SSAO is a key component in the inflammatory cascade leading to neutrophil infiltration and subsequent neurotoxicity in E2-treated OVX diabetics. The fact that VAP-1/SSAO activity has been reported to be enhanced in diabetic females (Yu et al., 2003) may have some relevance in this regard. Finally, gaining an understanding of the mechanisms responsible for this "transformation" of estrogen from a neuroprotective to a neurotoxic agent may provide clues that could help to explain the results of recently published clinical trials (reviewed by Brass, 2004) showing a lack of benefit with respect to stroke of hormone replacement therapy in postmenopausal females.

Acknowledgments

We acknowledge the expert technical assistance of Susan Anderson, Dennis Riley, Shuhua Ye, Li Huang, and Andrew Miller.

References


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