Peripheral Inflammation Exacerbates Damage After Global Ischemia Independently of Temperature and Acute Brain Inflammation

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Background and Purpose—Concomitant infection can exacerbate damage caused by cerebral ischemia. However, the interaction between and relative importance of the febrile and inflammatory components of the immune response is still unknown.

Methods—Male Sprague-Dawley rats were subjected to a 2-vessel occlusion with hypotension, immediately followed by intraperitoneal injection of lipopolysaccharide or pyrogen-free saline.

Results—Inflammation immediately after 2-vessel occlusion exacerbated hippocampal cell loss at 3 days and enhanced anxiety-related behaviors in the elevated plus maze and open field. These effects were not associated with differences in body temperature changes or with hippocampal pro-inflammatory cytokine production or hippocampal microglial activation.

Conclusion—We show a previously undocumented dissociation between lipopolysaccharide-exacerbated damage after global ischemia in the rat and the temperature and acute brain immune response, indicating that the mechanism for enhanced lipopolysaccharide damage is hippocampal cytokine and temperature independent in this case. (Stroke. 2007; 38:1570-1577.)

Key Words: activated microglia ■ anxiety ■ cytokines ■ lipopolysaccharide ■ two-vessel occlusion

Acutely preceding or concomitant infection can be a risk factor in humans for a cerebral infarct\(^1\,2\) and may lead to a poorer prognosis in the long-term.\(^3\,4\) Macko et al\(^1\) reported a high (35%) prevalence of infection or inflammation within the seven preceding days in patients presenting with a cerebral infarct, whereas similar reports have suggested that an infection up to 1 month before ischemia may precipitate or exacerbate it.\(^4\,5\) A cerebral infarct is likely, therefore, to occur on the background of an infection, and it is essential that we understand the impact this may have.

Body temperature is a major factor in neuronal survival after cerebral ischemia, with hypothermia being neuroprotective and hyperthermia being damaging.\(^6\,7\) Indeed, Thornhill and Asselin\(^8\) have demonstrated a greater degree of global ischemia-induced histological damage with a previous infection in only those rats in which fever developed. However, another aspect of the inflammatory response, potentially equally important in exacerbating damage after cerebral ischemia, is the production of pro-inflammatory cytokines. Pro-inflammatory cytokines are important mediators of inflammation and injury and are known contributors to excitotoxic damage.\(^11\) In humans the plasma cytokine status soon after a stroke can be correlated with long-term clinical outcome\(^12\,13\) and, in animal models, intracerebroventricular administration of antagonists, such as IL-1-ra, has been shown to have therapeutic potential.\(^15\)

Given the potential importance of cytokine production in ischemic cell damage, it is possible that the previously reported enhanced cell damage with a prior infection is attributable to alterations in central inflammatory processes. In this study we tested the hypothesis that a neuroimmune challenge concomitant with cerebral ischemia would exacerbate the damage caused by the latter and that this would be associated with an enhanced febrile and central inflammatory response. In addition to hippocampal cell survival and acute changes in temperature, cytokines, and activated microglia after a global ischemia and lipopolysaccharide (LPS) or saline, we also examined anxiety-related behaviors. The latter is a little-studied element of damage after cerebral ischemia in animal models, yet humans surviving cerebral ischemia have an increased incidence of anxiety,\(^16\,17\) which may severely impact on their quality of life.

Materials and Methods

Animals, Surgery, Immune Challenge

We conducted these experiments on 28 male Sprague-Dawley rats (Charles River; Montreal, Canada), \(~\text{300 grams, in accordance with the Canadian Council on Animal Care regulations and approved by the University of Calgary Animal Care Committee.}\)

Using halothane anesthesia (induced at 4%, maintained at 2%) we implanted temperature data loggers (SubCue) into the abdominal cavity under sterile conditions, as described previously.\(^18\) Animals

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were thereafter housed separately. After 4 days the rats were fasted for \( \approx 18 \) hours overnight to normalize blood glucose.\(^{19}\) We then performed a 2-vessel occlusion (2VO) as previously described.\(^{20}\) Briefly, we used sterile surgical techniques to occlude both common carotid arteries simultaneously for 15 minutes, maintaining blood pressure between 45 and 50 mm Hg with fine adjustments of the halothane anesthetic. During surgery brain temperature, estimated using a thermocouple in the ear, was maintained at 37.0±0.3°C using an overhead heating lamp. Immediately after surgery we injected either 50 \( \mu \)g/kg LPS (Escherichia coli; intraperitoneally; serotype 026:B6; Sigma, St. Louis, Mo) or an equivalent volume of pyrogen-free saline. The rats were then returned to their home cages and allowed to recover undisturbed.

Open Field
On day 3 after the 2VO we assessed the rats in the 3-day experiment for activity and anxiety using a mini open field paradigm as described previously.\(^{21}\) Each animal was observed for 10 minutes for locomotion (lines crossed), rearing (instances), grooming (instances and time spent), and number of fecal pellets.

Social Interaction
Immediately after the open field test we introduced an untreated unfamiliar male Sprague-Dawley rat of approximately the same weight (\( \pm 5 \) grams) to each treatment animal. We then assessed the treatment animal for 10 minutes for instances of 13 types of social behavior, an increase in which is thought to reflect a reduction in anxiety.\(^{22}\) Each behavior was scored separately and grouped as indicated: sniffing of the tail or undercarriage (“anogenital sniffing”); sniffing of the flank, nape, face (nose to nose), or body (“body sniffing”); crawling under or over (“active body contact”); brushing past or sitting alongside (“passive body contact”); following; allo-grooming; and boxing. We also scored the rats for instances of and time spent self-grooming.

Elevated Plus Maze
Approximately 1.5 hours after the social interaction test, we also assessed activity and anxiety-related behaviors for 10 minutes in the elevated plus maze (EPM), as previously described.\(^{22}\) We scored the rats for latency to move into an arm and number of entries into an open arm (expressed as a percentage of the total exploration time spent in either the open or closed arms). We also recorded number of “head dips” over the sides or ends of the arm, rearing, and grooming.

All behavioral tests were conducted during the light phase of the light/dark cycle. Testing equipment was thoroughly cleaned between animals.

Perfusions
At 4 hours or 3 days after the 2VO, the rats were deeply anesthetized with sodium pentobarbital. The rats euthanized at 3 days were perfused with 4°C phosphate-buffered saline via the left cardiac ventricle, followed by 10% paraformaldehyde. During surgery brain temperature, estimated using a thermocouple in the ear, was maintained at 37.0±0.3°C using an overhead heating lamp. Immediately after surgery we injected either 50 \( \mu \)g/kg LPS (Escherichia coli; intraperitoneally; serotype 026:B6; Sigma, St. Louis, Mo) or an equivalent volume of pyrogen-free saline. The rats were then returned to their home cages and allowed to recover undisturbed.

Cytokine Assessment
Hippocampi were homogenized and proteins extracted. Standard enzyme-linked immunosorbent assay kits were used to assess concentrations of TNF-\( \alpha \), IL-6, and IL-1\( \beta \) (Biosource International Inc., Camarillo, Calif) from these and the plasma samples. The inter-assay variabilities for these assays were, respectively, 2.6 to 2.7, 3.7 to 4.9, 6.7 to 8.2% coefficient of variance, intra-assay variabilities 3.5 to 3.9, 5.9 to 9.9, 8.7 to 9.7% coefficient of variance, and lower limit of detection \(< 8 \) pg/mL. All samples from each experiment were assayed together in duplicate. Brain concentrations were corrected for amount of protein in the sample.

Histology
Fixed brains were embedded in paraffin and 10-\( \mu \)m coronal sections cut and mounted onto polylysine-coated slides. The extent of ischemic injury at 3 days was quantified by histological analysis of remaining hippocampal and central amygdala cells in Harris hematoxylin and eosin-stained coronal sections. Neuronal survival was evaluated by an experimenter, blind to the rats’ treatment groups, in the CA1 and the CA3 subfields of the hippocampus from and caudal to –4.2 mm relative to bregma, and in the central amygdala from and caudal to –2.3 mm relative to bregma, in 4 10-\( \mu \)m sections 120 \( \mu \)m apart. These 4 values were summed to obtain a total for each region.

Numbers of degenerating cells were quantified by analysis of sections, adjacent to those used for hematoxylin and eosin, stained with Fluoro-Jade.\(^{23}\) Degenerating cells, those that fluoresced green under a FITC filter, were counted in the CA1, CA3, and dentate gyrus subfields of the hippocampus as described.

Immunohistochemistry
Coronal sections adjacent to those used for Fluoro-Jade, and equivalent for the 4-hour groups, were immunolabeled for the presence of mononuclear phagocytes (microglia/macrophages)\(^{24}\). Briefly, sections were incubated in primary Iba1 antibody (overnight; 1:400; rabbit; Wako Chemicals USA, Inc., Richmond, Va), followed by the secondary antibody (2 hours; 1:400: donkey anti-rabbit IgG [CY3]; Jackson ImmunoResearch Laboratories, West Grover, Pa).

Because of the extremely high numbers of labeled microglia in the hippocampus, we analyzed only a subsection of each hippocampal section and only one section per animal. Thus, 3 grids, each encompassing 25×25 \( \mu \)m of hippocampal section, were examined at \( \times 40 \) magnification for numbers of activated and inactive microglia. For each hippocampal section, the grids were positioned at: (1) the most medial aspect of the CA1 region; (2) the apex, or most dorsal point, of the CA1; and (3) the most lateral aspect of the CA1 where CA3 cells become apparent. We examined the CA1 in this way because this was the area where we saw significant differences in cell survival at 3 days. Activated microglia were distinguished from inactive by the presence of shorter less ramified processes and by their perikaryal hypertrophy and ameboid appearance.\(^{24}\)

Data Analysis
All scores were compared using 2-tailed Student unpaired \( t \) tests. \( F \) tests for heterogeneity of variance were assessed and appropriate \( t \) tests conducted. For temperature, the value at each 5 minutes interval was used to calculate a baseline for the hour before surgery. The mean change from this baseline was then calculated per hour for each animal for each of 1 to 2 hours and 3 to 6 hours after the surgery. Temperature to CA1 cell comparisons were performed using a Pearson’s correlation coefficient comparing peak temperature averaged over 15 minutes with total CA1 counts for each animal. In each case, statistical significance was assumed when \( P<0.05 \). Data are presented as mean±SEM.

Results
Histology
LPS injection (\( n=6 \)) immediately after the 2VO resulted in significantly fewer surviving cells in the CA1 hippocampus than in saline-treated rats (\( n=7 \)) when assessed 3 days after surgery (\( t=2.46, P=0.03 \); Figure 1A, 1C, and 1D). No differences were seen between rostrocaudal levels for either of the groups indicating no anterior/posterior differences in vulnerability. No significant differences were seen in cell survival in the CA3 region (\( t=1.7, P=0.12 \); Figure 1B) or in
the central amygdala (2VO-saline=751.4±47.9; 2VO-LPS=656.7±29.1; t=1.62, P=0.13). LPS after the 2VO was also associated with a tendency to greater numbers of degenerating neurons in the CA1 (t=1.59, P=0.14; Figure 1E), CA3 (t=1.80, P=0.099; Figure 1F), and dentate gyrus (2VO-saline=98.5±45.3; 2VO-LPS=232.7±63.2; t=1.77, P=0.10). No degenerating neurons were seen in the central amygdala or elsewhere in the sections examined except some scattered in the cortex and the internal capsule.

Open Field
An assessment of anxiety and activity in the mini open field revealed no significant differences between saline- and LPS-treated rats in any of the locomotor (2VO-saline=36.8±7.1; 2VO-LPS=39.8±7.8 lines crossed) or rearing (2VO-saline=32.9±5.0; 2VO-LPS=31.7±7.8 instances) parameters assessed, or in numbers of fecal pellets (2VO-saline=4.3±1.1; 2VO-LPS=3.8±1.4), but did reveal an interesting difference in their grooming behavior. 2VO-LPS animals had a significantly shorter latency to groom than the saline-treated controls (t=3.75, P=0.04; Figure 2A) and spent significantly more of their time grooming (t=2.71, P=0.02; Figure 2B), although no differences were seen in numbers of bouts of grooming (2VO-saline=2.6±0.8; 2VO-LPS=3.3±0.8). We did not observe any interruptions in the typical cephalocaudal grooming pattern. Indeed, most grooming bouts were targeted only at the head.

Social Interaction
No significant differences were seen between the groups in any of the 13 behaviors scored in the social interaction test. Grouping these individual behaviors into broader categories also failed to
highlight any significant differences between treatment groups (Table). We also assessed time spent anogenital sniffing and saw no differences between the groups (2VO-saline = 94.8 ± 15.7 seconds; 2VO-LPS = 116.6 ± 16.7 seconds).

We did see a tendency for the LPS-treated rats to spend more time self-grooming, but this difference was not statistically significant (2VO-saline = 5.8 ± 2.2 seconds; 2VO-LPS = 9.1 ± 4.6 seconds; t = 0.68, P = 0.51).

**Elevated Plus Maze**
Despite generally low levels of total exploration in the EPM in both groups, the LPS-treated rats displayed significantly less open arm exploration as a percentage of total compared with controls (t = 2.60, P = 0.02; Figure 2C). Total exploration (number of arm entries) was not significantly different between groups (2VO-saline = 23.4 ± 4.7; 2VO-LPS = 18.1 ± 2.4; t = 0.86, P = 0.41), and this finding therefore likely reflects an increase in anxiety rather than a general impairment of locomotor activity. No differences were seen between the groups in latency to move into an arm, head dipping, rearing, or grooming (data not shown).

**Body Temperature**
Body temperatures measured during and after the 2VO indicate no effect of LPS (n = 6) compared with saline (n = 8) on the temperature changes associated with the surgery. During surgery, body temperatures were maintained between 36.0°C and 36.5°C with no significant difference between the groups. After the 2VO, the rats became hypothermic for ≈2 hours, after which the temperatures recovered to become slightly hyperthermic compared with starting temperatures (Figure 3A and 3B).

Despite a tendency for LPS to cause a greater hyperthermic response 3 to 6 hours after the surgery (Figure 3A and 3B), no significant differences in mean or peak temperatures (averaged over 15 minutes) were observed between the 2 groups (peak: 2VO-saline = 37.5 ± 0.5; 2VO-LPS = 37.8 ± 0.2°C), and there was no correlation between individual animals’ temperature changes and CA1 hippocampal cell survival (Figure 3C). Furthermore, temperature and circadian temperature rhythmicity remained the same between the groups for the duration of the experiment (Figure 3D). Therefore, an enhanced febrile response is not necessary for the LPS-enhanced damage we see after global ischemia.

**3-Day Activated Microglia**
Counts of activated microglia in subsections of the CA1 region of the hippocampus revealed no significant differences between saline and LPS-treated groups either in absolute numbers of activated microglia (2VO-saline = 102.4 ± 13.1; 2VO-LPS = 109.0 ± 9.5), or when expressed as a ratio to inactive cells (Figure 4). Qualitative analysis of other regions of the hippocampus also revealed no differences between the groups. These data indicate the absence of a lasting increased inflammatory response after LPS.

**Acute Changes in Hippocampal and Plasma Cytokines and Activated Microglia**
We found no differences between the saline-treated (n = 6) and LPS-treated (n = 7) groups in levels of hippocampal pro-
inflammatory cytokines 4 hours after the 2VO (Figure 5A, 5B, and 5C), indicating that an enhanced acute central inflammatory response is unlikely to be responsible for increased damage at 3 days in LPS-treated rats. There were also no differences in numbers of activated microglia in the CA1 hippocampus 4 hours after the 2VO and LPS/saline injection (Figure 5D). As expected, LPS did increase circulating pro-inflammatory cytokines and this was still evident at 4 hours (TNF-α: t = 3.40, P = 0.009; IL-6: t = 2.30, P = 0.047; Figure 5E and 5F). Plasma IL-1β was not significantly increased at 4 hours with LPS (2VO-saline = 29.7 ± 5.6; 2VO-LPS = 28.8 ± 4.5 pg/mL; t = 0.13, P = 0.90).

Discussion

In this investigation we describe 3 particularly important findings. First, we show here that a systemic inflammation occurring in conjunction with a global ischemic insult can lead to more neuronal damage in the hippocampus, particularly in the CA1 region, than would occur with the global ischemia alone. Second, we show that this enhanced CA1 damage is associated with more anxious behavior in some tests of anxiety. Third, we demonstrate a dissociation between this enhanced damage and both the temperature and acute brain immune response.

Perhaps the most interesting finding to note from this study is the dissociation between this LPS-exacerbated damage and the acute immune response. In an exciting compliment to findings from previous investigations, our rats were not significantly febrile after LPS and a 2VO despite sustaining more hippocampal CA1 damage at 3 days than controls. Thornhill and Asselin10 have found with LPS 5 hours before global hemispheric hypoxic ischemia, producing unilateral ischemia, that only those animals that developed fever (temperature >38°C) after LPS had hippocampal damage worse than saline-treated controls with cerebral ischemia. Nonfebrile LPS-injected rats were not different from controls. In response to LPS immediately after the 2VO, our rats’ temperatures did not exceed the 38°C described as the critical level at which hyperthermia exacerbates ischemia damage, and yet we still see exacerbated damage. It is likely that, in our study, the acute effects of the global ischemia acted to dampen the normal LPS-induced febrile response, but that an enhanced inflammatory response was still mounted. One should note that Thornhill and Asselin used Long Evans rats in their study, which appear to have a very different febrile response from that of Sprague-Dawleys. Whereas Thornhill saw a peak febrile response at 5 hours, adult Sprague-Dawleys show a peak fever at ~2 hours.25 The finding that nonfebrile rats do not display exacerbated damage after LPS and ischemia probably reflects differences in the strain, timing, and model used. The important point is that we find it is not necessary to develop a fever with an infection to have enhanced damage, neurological and behavioral, after a global cerebral ischemia.
Circulating cytokines have, at least in humans, been correlated with outcome after a stroke,12–14 and we did see, as expected, an increase in plasma TNF-α and IL-6 in the LPS-treated rats at 4 hours after LPS. Central pro-inflammatory cytokines consequent to peripheral inflammation may also be important in cerebral ischemia.33 We therefore examined whether there was an elevation of pro-inflammatory cytokines in the brains of the 2VO-LPS animals that might account for the increased damage. No differences were found. However, we cannot exclude that there may be differences in brain anti-inflammatory cytokines or in the cellular responses to cytokines between the 2 groups.

Thus, the mechanisms linking infection and cerebral ischemia remain unclear.

Activated inflammatory cells such as microglia are among the first to respond to a brain insult and have been implicated in ischemic injury.26 These cells are a source of cytokines and other inflammatory mediators34 and, when activated after a cerebral ischemia, accelerate injury via an overexpression of inducible nitric oxide synthase and production of cytotoxic nitric oxide.27 In accordance with the similar levels of hippocampal cytokines between the groups, we also saw similar levels of activated microglia. However, it is possible that the 2VO activated maximal numbers of microglial cells but that LPS had an additional cytokine- and temperature-independent effect on inducible nitric oxide synthase in each cell. LPS is known to activate the transcription factor nuclear factor κB and thereby activate the inducible nitric oxide synthase promoter,28 and may therefore contribute directly to inducible nitric oxide synthase induction and cell damage.

One should also note that, in our investigation, we measured pro-inflammatory cytokines and activated microglia at only one acute time point. It is possible that these markers of inflammation were differentially affected at different time points. It is also possible that the findings presented here reflect accelerated rather than increased damage, thus assessment of cell death at later time points may have revealed identical patterns of injury. Our assessment of degenerating cells goes some way to disputing this suggestion, however, because, in addition to having fewer surviving cells in the CA1, the LPS-treated rats also had a tendency to more degenerating cells, indicating that such increased damage may be ongoing.

Human patients with a cerebral infarct have a high likelihood of developing anxiety and depression during recovery.16,17 It is therefore important to consider these affective disorders in animal models of cerebral ischemia. Rat anxiety has been shown to be strongly reflected in alterations in time spent grooming and in grooming patterns.29 For instance, in a high light situation, commonly held to be stressful for a rat,30 rats display significantly shorter latencies to groom, more bouts of grooming, and spend more time grooming. Here we observed a significant increase in the time spent grooming in the mini open field, as well as a reduction in the latency to groom, in rats that had LPS immediately after the 2VO compared with saline-treated controls. We also found another indicator of anxiety in these LPS-treated rats in the EPM: they had fewer entries into the anxiety-provoking open arms31 as a percentage of the total exploration of the EPM than did the saline-treated controls. In contrast to our effects on anxiety-linked behaviors, we did not see any effect on locomotor activity. In the open field and EPM both groups explored the area to the same degree, an important indicator that motor control was not adversely affected. These findings therefore provide strong evidence that an inflammation occurring at the same time as a global ischemia can alter behavioral outcomes, particularly those associated with increased anxiety.

Of the higher brain regions associated with anxiety, the amygdala is one we have shown to be altered with global cerebral ischemia in rats that received an immune challenge as neonates.20 However, we observed no substantial amygd-
damage in either saline or LPS-treated 2VO rats. It is possible that the effects on grooming and EPM open arm entry seen in this study may be caused by alterations in the amygdala, but that a histological analysis of intact and degenerating cells was not sufficient to reveal this. The amygdala neurons may have remained ostensibly intact while being functionally abnormal because of changes in synaptic density or receptor function, as has been shown occurs with other populations of cells. It is also likely that other brain regions are involved. Both the neostriatum and the hypothalamus have been associated with grooming, albeit with the sequence or “syntactical” organization of the grooming pattern in which we did not see any differences. Unfortunately, little evidence is available as to the brain regions responsible for initiating grooming.

Open arm entry in the EPM is probably governed by brain regions traditionally involved in stress circuitry such as the paraventricular nucleus of the hypothalamus and associated brain areas. It would be interesting to examine some of these regions in more detail to determine whether they incurred any additional damage with the LPS.

The findings outlined in the present investigation have important clinical relevance in that they highlight the role of infection and inflammation in outcomes after a cerebral ischemia. We have shown that neurological and behavioral outcomes are worsened when a global ischemia occurs in the...
presence of even subfebrile inflammation. Thus, the presence of infection at the time of cerebral infarct, even in the absence of a fever, is an important factor that needs to be assessed when considering treatments.

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Disclosures
None.

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