Anxiety After Cardiac Arrest/Cardiopulmonary Resuscitation
Exacerbated by Stress and Prevented by Minocycline

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Background and Purpose—Stress is an important risk factor for cardiovascular disease; however, most of the research on this topic has focused on incidence rather than outcome. The goal of this study was to determine the effects of prior exposure to chronic stress on ischemia-induced neuronal death, microglial activation, and anxiety-like behavior.

Methods—In Experiment 1, mice were exposed to 3 weeks of daily restraint (3 hours) and then subjected to either 8 minutes of cardiac arrest/cardiopulmonary resuscitation (CA/CPR) or sham surgery. Anxiety-like behavior, microglial activation, and neuronal damage were assessed on postischemic Day 4. In Experiment 2, mice were infused intracerebroventricularly with minocycline (10 μg/day) to determine the effect of inhibiting post-CA/CPR microglial activation on the development of anxiety-like behavior and neuronal death.

Results—CA/CPR precipitated anxiety-like behavior and increased microglial activation and neuronal damage within the hippocampus relative to sham surgery. Prior exposure to stress exacerbated these measures among CA/CPR mice, but had no significant effect on sham-operated mice. Treatment with minocycline reduced both neuronal damage and anxiety-like behavior among CA/CPR animals. Anxiety-like behavior was significantly correlated with measures of microglial activation but not neuronal damage.

Conclusions—A history of stress exposure increases the pathophysiological response to ischemia and anxiety-like behavior, whereas inhibiting microglial activation reduces neuronal damage and mitigates the development of anxiety-like behavior after CA/CPR. Thus, modulating inflammatory signaling after cerebral ischemia may be beneficial in protecting the brain and preventing the development of affective disorders. (Stroke. 2009;40:3601-3607.)

Key Words: anxiety ■ cardiac arrest ■ inflammation ■ microglia ■ stress

Exposure to stressful life events can increase the probability of cardiac arrest (CA),1,2 and may complicate recovery. Indeed, prolonged exposure to stress or glucocorticoids decreases neuronal viability and increases microglial reactivity.3,4 Priming of microglia by stress could impact their response to central nervous system injury; on activation, microglia release proinflammatory cytokines, proteolytic enzymes, and nitric oxide and can become phagocytic.5,6 Global ischemia potently activates microglia,7,8 but the effects of prior exposure to stressors on postischemic microglial activation have not been determined.

The role of microglial activation in modulation of affect behavior also is not well characterized, although stimulating reactive microglia in otherwise healthy rats increases anxiety-like behavior.9 Given that increases in anxiety are reported in both people10,11 and rodents12–14 that survive cardiac arrest, and that cerebral ischemia reliably activates microglia, there may be a role for posts ischemic microglial activation in the development of anxiety after cardiac arrest. Suppressing microglial activation with minocycline reduces neuronal damage in other models of cerebral ischemia15,16 and ameliorates anxiety-like behavior after neonatal hypoxia-ischemia.17 The current study examines the influence of chronic stress on cardiac arrest/cardiopulmonary resuscitation (CPR)-induced microglial activation, neurodegeneration, and anxiety-like behavior, and also determines the necessity of activated microglia for the generation of cardiac arrest/CPR-induced anxiety-like behavior.

Materials and Methods

Animals

Adult male C57BL/6 mice (Charles River, Portage, Mich) were randomly assigned to groups. Experiment 1 consisted of 4 groups: 1) SHAM (n=8); 2) SHAM+STRESS (n=9); 3) CA/CPR (n=8);
and (4) CA/CPR + STRESS (n=9). In Experiment 2, mice were treated intracerebroventricularly with minocycline (MIN) or its vehicle (VEH); they were assigned to: (1) SHAM + VEH (n=5); (2) SHAM + MIN (n=5); (3) CA/CPR + VEH (n=7); or (4) CA/CPR + MIN (n=7). This study was approved by The Ohio State University Animal Care and Use Committee and conforms to guidelines provided by the National Institutes of Health for the care and use of animals. All surgeries were conducted under sterile conditions and mice were not returned to the colony after surgery until mobile. They were visually monitored twice daily afterward.

Restrain
Mice were placed in well-ventilated polypropylene tubes (9.7 cm long, 2.8 cm inside diameter) that allow postural adjustments but not turns for 3 hours/day for 3 weeks during the light cycle. The final restraint session occurred 24 hours before surgery. This method of daily restraint reliably elicits a corticosterone response for up to 6 weeks of exposure.

Cardiac Arrest Procedure
Mice were anesthetized with halothane and intubated. Brain and core body temperature were assessed using temperature probes placed in the temporalis muscle (methodological validation) and rectum, respectively. Head and body temperature were independently controlled through water-filled coils. A PE10 catheter was inserted into the right jugular vein for potassium chloride and epinephrine administration. A blood pressure transducer (Columbus Instruments, Columbus, Ohio) was connected to a right femoral artery cannula. Mice were ventilated at a tidal volume of 120 μL and a respiratory rate of 160 breaths per minute (Columbus Instruments). Blood pressure and temperatures were recorded at 1-minute intervals during a 10-minute acclimation period (Figure 1). Body temperature was decreased to 27°C to prevent peripheral organ damage. Head temperature was maintained at 37°C. To induce cardiac arrest, cold potassium chloride (50.0 μL, 0.5 mol/L, 4°C) was infused and the mouse was detached from the ventilator. Rewarming began when body temperature reached 27°C after approximately 4 minutes of arrest. At 7 minutes 45 seconds into the arrest period, the mouse was reattached to the ventilator and began to receive 100% oxygen. At 8 minutes, 8 μg of epinephrine in 0.5 mL saline was injected and chest compressions (approximately 300/minute) initiated. Additional epinephrine was administered in increments of 0.5 μg/30 seconds until mice were resuscitated. Mice were maintained on 100% oxygen for 15 minutes, then extubated, followed by the removal of catheters and suturing of wounds.

The surgical preparations, anesthetic exposure, and temperature modulation described were similar for SHAM mice, except that they received injections of isotonic saline instead of potassium chloride and epinephrine and were not given chest compressions.

Minocycline
Minocycline, a tetracycline derivative with anti-inflammatory properties, prevents microglial activation after neurological insults. In Experiment 2, a stereotaxic apparatus was used to implant a cannula into the left lateral ventricle of anesthetized mice (isoflurane) 3 days before CA/CPR (cannula position: +0.02 posterior and −0.95 lateral to bregma, extending 2.75 mm below the skull; Plastics One, Roanoke, Va). The cannula was connected by tubing to an Alzet minipump (Model 1002; Durect, Cupertino, Calif) that was implanted subcutaneously in the scapular region and delivered artificial cerebrospinal fluid (the vehicle) or minocycline at a rate of 0.25 μL/h. The minocycline group received 10 μg of the drug per day beginning 12 hours before CA/CPR; the minipump tubing and cannula were primed to deliver artificial cerebrospinal fluid for the first 2.5 days after implantation. The cannula and pump were implanted during a single surgery, 3 days before CA/CPR or SHAM, in an attempt to minimize surgical stress. Cannula placement was verified with cresyl violet staining after tissue collection.

Behavioral Testing
Total locomotor activity, rearing, and central tendency were assessed for 60 minutes in an open field (40 cm × 40 cm × 37.5 cm; San Diego Instruments, Calif) 1 day before surgery and 4 days after surgery for both experiments. Central tendency is the percent time spent in the 90-cm2 zone in the center of the apparatus. Habituation can occur with repeated exposure to the open field as the novelty of the open field diminishes over time, but the anxiogenic properties of the center do not decrease. Because the CA/CPR + STRESS group in Experiment 1 and the CA/CPR + MIN group in Experiment 2 had 2 levels of treatment, the use of a baseline measure (after stress or minocycline but before CA/CPR) allowed within-subject control for the combined condition when using analysis of variance with repeated measures.
Histology
On Day 5 postsurgery, mice were transported one at a time from the colony, deeply anesthetized (pentobarbital) and perfused with 0.1 mol/L phosphate-buffered saline followed by perfusion, then post-fixing, with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. Brains were cryoprotected in 30% sucrose in 0.2 mol/L phosphate-buffered saline. The region containing the hippocampus was cut into 10-µm sections and thaw-mounted onto slides. The slides were incubated in 0.2 mol/L phosphate buffer with 0.1% Triton X-100 and blocked with 4% rabbit serum. Antimouse MAC-1 (1:100; Serotec, Oxford, UK) was then added to the slides and incubated overnight at 4°C. Then, the slides were rinsed in 0.5 mol/L Tris buffer and incubated overnight at 4°C in biotinylated rabbit antirat (Vector, Burlingame, Calif) in 4% rabbit serum. Slides were then rinsed in 0.5 mol/L Tris buffer and visualized with diaminobenzidine and counterstained with 0.1% cresyl violet. Finally, slides were dehydrated through a series of graded ethanol solutions followed by xylene and then coverslipped.

Histological measures were collected by an individual who was not aware of group assignment. The degree of microglia activation was qualitatively analyzed by the summation of 2 scores. The first score was assigned based on the degree of microglial activation in the hippocampus. The scale was as follows: 0 = no glial activation; 1 = mild CA1/CA2 glial activation; 2 = moderate glial activation throughout the hippocampus; and 3 = pronounced glial activation throughout the hippocampus. The second score described glial activation outside the hippocampus (eg, cortex or caudate/putamen) and was as follows: 0 = no glial activation; 1 = mild activation; 2 = pronounced activation; and 3 = pronounced activation in more than one region. The 2 scales were summed for each mouse and then group medians determined. Alternate sections were stained with hematoxylin and eosin, dehydrated, and coverslipped. Pyknotic cells were counted at 600X in the dorsal hippocampus proper and dentate gyrus of each hemisphere and then averaged.

Statistical Analyses
Behavioral parameters were assessed by 3-way repeated-measures analyses of variance using the factors of time (pre-CA/CPR versus post-CA/CPR), surgery (SHAM versus CA/CPR), and stress (control versus restraint) in Experiment 1 and the factors of time (pre-CA/CPR versus post-CA/CPR), surgery (SHAM versus CA/CPR), and drug (VEH versus MIN) in Experiment 2. Neuronal damage was assessed by 2-way analyses of variance using the factors of surgery (SHAM versus CA/CPR) and stress (control versus restraint) in Experiment 1 and the factors of surgery (SHAM versus CA/CPR) and drug (VEH versus MIN) in Experiment 2. Post hoc analyses were used to further distinguish among groups, and all differences were considered statistically significant if P<0.05. Microglial activation was analyzed using a Mann-Whitney rank sum test because the data did not meet the criteria for parametric analysis. Correlations between microglial activation or cell death and central tendency were assessed using the nonparametric Spearman rank order correlation. Alpha was 0.05 for all parametric and nonparametric statistical analyses.

Results
Surgical Parameters
Neither stress nor minocycline altered blood pressure, temperature, surgical time, or resuscitation time (Figure 1). As expected, CA/CPR decreased mean blood pressure as compared with SHAM during the period between potassium chloride and epinephrine administration (F[3,56]=104.3, P<0.05; Figure 1A). Temporalis temperature was higher among CA/CPR than SHAM mice (F[3,56]=34.1, P<0.05; Figure 1B). Core body temperature was lower among CA/CPR than SHAM mice (F[3,56]=9.8, P<0.05; Figure 1C).

Behavioral Testing
In Experiment 1, groups did not differ in general locomotor activity during presurgical or postsurgical testing (P>0.05), and there was no significant change in total activity between these 2 time points for any group (P>0.05). In contrast, central tendency was similar among groups during presurgical testing but reduced among CA/CPR groups during postsurgical testing (F[1,33]=57.23, P<0.05; Figure 2A). Furthermore, CA/CPR + STRESS further increased anxiety-like behavior relative to the CA/CPR group (P<0.05). Restraint did not impact anxiety-like behavior among SHAMs (P>0.05).

In Experiment 2, there were no significant pre- or postsurgical group differences in general locomotor activity or rearing (P>0.05) and no significant change in these measures between pre- and postsurgical testing for any group (P>0.05). There were no group differences in central tendency at presurgical testing (P>0.05), but the CA/CPR + VEH group reduced central tendency relative to the other 3 groups on postsurgical Day 4 (F[1,19]=13.59, P<0.05). In contrast, central tendency was similar for CA/CPR + MIN and the 2 SHAM groups (F[11]=2.55, P<0.05;
Minocycline also increased central tendency in SHAM mice compared with the vehicle group ($F[1,9]=16.053$, $P<0.05$; Figure 2B).

**Microglial Analysis**

In Experiment 1, there was no microglial activation in SHAM brains (Figure 3A–B). CA/CPR caused activation of microglia, primarily in the hippocampus (Figure 3C–D). CA/CPR+STRESS increased both the level of microglial activation and its spatial distribution relative to CA/CPR ($t[11]=87.5$, $P<0.05$; Figures 3E–F and 4).

In Experiment 2, CA/CPR activated microglia in both vehicle and minocycline-treated mice ($F[3,22]=13.6$, $P<0.001$; Figures 4B and 5) relative to SHAM. Post hoc analysis revealed that CA/CPR+VEH mice had significantly greater microglial activation than SHAM mice, but microglial activation was significantly reduced among CA/CPR+MIN mice, which did not differ from SHAM ($P>0.05$).

Spearman rank order correlation demonstrated that microglial activation was correlated with anxiety-like behavior ($r^2=-0.45$, $P<0.05$).

**Assessment of Pyknotic Cells**

In Experiment 1, there was no cell death in SHAM brains. CA/CPR caused a significant increase in pyknotic cells in the hippocampus (Figure 6A; $H[2]=17.8$, $P<0.05$) relative to SHAM. CA/CPR+STRESS increased rating of pyknotic cells in the hippocampus relative to CA/CPR alone ($t[15]=2.281$, $P<0.05$).

In Experiment 2, there was no cell death in SHAM brains, and CA/CPR significantly increased the number of pyknotic cells ($F[2,23]=21.820$, $P<0.05$; Figure 6B) in the hippocampus. A post hoc analysis revealed that CA/CPR+VEH mice had more pyknotic cells than SHAM operated mice; however, CA/CPR+MIN significantly reduced the number of pyknotic cells relative to CA/CPR+VEH ($P<0.05$).

Spearman rank order correlation demonstrated that the number of pyknotic cells was not correlated with anxiety-like behavior ($P>0.05$).

**Discussion**

Collectively, these data demonstrate that a history of stress exposure exacerbates post-CA/CPR anxiety-like behavior and augments microglial activation and neuronal cell death.
In addition, ischemia-induced anxiety-like behavior can be prevented by minocycline, which attenuates both microglial activation and neuronal death. Anxiety-like behavior was significantly correlated with microglial activation, but not neuronal damage. These data confirm that surviving cardiac arrest precipitates the development of anxiety and suggests that minocycline administration may be an effective treatment.

These data confirm previous studies indicating that global ischemia induced in rodents by CA/CPR results in neuronal cell death, microglial activation, and increased anxiety-like behavior. However, the indication that these 3 measures are exacerbated when exposure to chronic restraint precedes CA/CPR provides new evidence for the importance of stress as a risk factor for cardiovascular disease. Stress is a known risk factor for the onset of CA, but the current data suggest that it also affects outcome among survivors. Prior exposure to stress altered the pattern of microglial activation after CA/CPR but not SHAM surgery, thereby suggesting that the morphological effects of stress on microglia are only apparent after injury. Furthermore, stress altered the spatial distribution of activated microglia after CA/CPR; in the absence of prior stress, microglial activation occurred exclusively in the hippocampus with preferential involvement of the pyramidal cell layer at the border of CA1 and CA2. In contrast, the combination of stress and CA/CPR led to
additional microglial activation in the cortex and caudate/putamen. Although the mechanisms may differ, a similar negative effect of stress on neuronal damage and cognitive function has been demonstrated after experimental stroke.23

The association of increased microglial activation with increased neuronal death and anxiety-like behavior after CA/CPR in the current study suggests that activated microglia are contributing to early postischemic neuropathology and behavioral changes. However, because microglia can improve survival of metabolically impaired neurons under other circumstances, the relationship between microglia and neurons may be dynamic. Whether the role of microglia in brains exposed to CA/CPR changes over time, as the initial wave of neuronal death passes, remains to be determined.

Consistent with previous reports,17,25 minocycline administration inhibited microglial activation after CA/CPR, decreased cell death, and prevented post-CA/CPR anxiety-like behavior. However, the correlation between microglial activation and anxiety-like behavior suggests that the anxiolytic effects of minocycline may be associated with inhibition of microglial activation. The absence of a significant correlation between neuronal damage and behavioral outcome is consistent with previous studies in which cell death did not predict postischemic behavioral outcome.26 However, given that minocycline decreased both cell death and microglial activation after CA/CPR, we cannot rule out the potential for cell survival to contribute to the changes in behavior. Regardless of the precise mechanism, minocycline did effectively prevent post-CA/CPR anxiety-like behavior and decrease cell death.

**Summary**

Exposure to stress augments CA/CPR-induced anxiety-like behavior and increases microglia activation and neuronal death in the hippocampus. Minocycline, which has been effective in minimizing damage in a clinical stroke study,27 inhibited microglial activation, cell death, and anxiety-like behavior after CA/CPR in the current mouse study. These data complement a growing body of literature documenting the impact of neuroinflammation on affective behavior. Identifying the postischemic physiology that underlies changes in affective behavior is important for improving the quality of life of CA survivors.

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**Disclosures**

None.

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