Cinnamophilin offers prolonged neuroprotection against gray and white matter damage and improves functional and electrophysiological outcomes after transient focal cerebral ischemia*

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Objective: **We have previously shown that cinnamophilin ([8R, 8′S]-4, 4′-dihydroxy-3, 3′-dimethoxy-7-oxo-8, 8′-neolignan) exhibited potent antioxidant, radical-scavenging, and anti-inflammatory actions and reduced acute ischemic brain damage, even when it was given up to 6 hrs postinsult. Here, we characterized the long-lasting neuroprotection of cinnamophilin against gray and white matter damage and its beneficial effects on electrophysiological and functional outcomes in a model of stroke.**

Design: **Prospective laboratory animal study.**

Setting: **Research laboratory in a university teaching hospital.**

Subjects: **Adult male Sprague-Dawley rats (240–290 g).**

Interventions: **Under controlled conditions of normoxia, normocarbia, and normothermia, spontaneously breathing, halothane-anesthetized (1.0–1.5%) rats were subjected to transient middle cerebral artery occlusion for 90 mins. Cinnamophilin (80 mg/kg) or vehicle was given intravenously at reperfusion onset.**

Measurements and Main Results: **Physiological parameters, including arterial blood gases and cortical blood perfusion, somatosensory-evoked potentials, and neurobehavioral outcomes, were serially examined. Animals were euthanized at 7 days or 21 days postinsult. Gray matter and white matter (axonal and myelin) damage were then evaluated by quantitative histopathology and immunohistochemistry against phosphorylated component-H neurofilaments and myelin basic protein, respectively. After the follow-up period of 7 and 21 days, our results showed that cinnamophilin significantly decreased gray matter damage by 31.6% and 34.9% ($p < .05$, respectively) without notable adverse effects. Additionally, cinnamophilin effectively reduced axonal and myelin damage by 46.3–68.6% ($p < .05$) and 25.2–28.1% ($p < .05$), respectively. Furthermore, cinnamophilin not only improved the ipsilateral field potentials ($p < .05$, respectively), but also reduced the severity of contralateral electrophysiological diaschisis ($p < .05$). Consequently, cinnamophilin improved sensorimotor outcomes up to 21 days postinsult ($p < .05$, respectively).**

Conclusions: **Administration with cinnamophilin provides long-lasting neuroprotection against gray and white matter damage and improves functional and electrophysiological outcomes after ischemic stroke. The results suggest a need for further studies to characterize the potential of cinnamophilin in the field of ischemic stroke. (Crit Care Med 2011; 39:1130–1137)**

Key Words: acute stroke; neuroprotection; white matter damage; evoked potentials; functional recovery; and cinnamophilin

Ischemic stroke commonly affects cytoarchitectures, intercellular synapses, and functional circuitries of gray and white matter. Histologic assessment in experimental stroke is, however, dominated by staining of neuronal perikarya. Increasing evidence has indicated that the assessment of stroke therapies may need to evaluate both gray and white matter pathology (1, 2). Additionally, the assessments of neurobehavioral and electrophysiological outcomes are important in the translational research for ischemic stroke therapies, because functional improvements are the most important clinical end in the treatment of ischemic stroke. Furthermore, the long-lasting efficacy is critical in the assessment of putative neuroprotectants, because many therapies are known to protect against ischemic insults after a short term of recovery but have been proven ineffective with a long-term recovery period (3).

Cinnamophilin (CINN) ([8R, 8′S]-4, 4′-dihydroxy-3, 3′-dimethoxy-7-oxo-8, 8′-neolignan) isolated from *Cinnamomum philippinense* is a novel antioxidant and free radical scavenger (4, 5). The agent readily penetrates the blood–brain barrier with a slow decay in the brain (5). We have demonstrated that CINN (20–80 mg/kg) dose-dependently reduces acute brain damage by attenuating the *in situ* accumulation of superoxide anions and the immunoreactivity of the 4-hydroxynonenal and 8-hydroxy-2′-deoxyguanosine, markers of cellular membranous and DNA lipid peroxidation,

*See also p. 1230.

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respectively, after transient focal cerebral ischemia in rats (5). More recently, we have shown that CINN protects against ischemic stroke with a therapeutic window up to 6 hrs *in vitro* and *in vivo*. Additionally, we found that CINN had direct antioxidant effects by reducing the malondialdehyde levels in the ischemic brain tissues and the Fe^{3+}-induced lipid peroxidation in rat brain homogenate (6). Furthermore, CINN was found to display potent direct radical scavenging actions as assessed by 2, 2-diphenyl-1-picrylhydrazyl and 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt assays (6). The agent also exhibited potent anti-inflammatory actions by effectively inhibiting the myeloperoxidase activity in the ischemic brain tissues and the production of tumor necrosis factor-α, nitrite/nitrate as well as interleukin-6 (IL-6) in the lipopolysaccharide-stimulated RAW 264.7 and BV2 cells, respectively (6). Although some neuroprotective actions of CINN have been characterized at the acute stage of stroke, its long-lasting neuroprotection, particularly the ability to protect against gray and white matter damage and to improve electrophysiological and functional outcomes, remains to be clarified. Thus, we evaluated whether intravenous administration of CINN could protect against gray and white matter damage and, therefore, improve neurobehavioral and electrophysiological outcomes after two prolonged periods (7 and 21 days) of recovery after transient focal cerebral ischemia in rats.

MATERIALS AND METHODS

All procedures performed were approved by the Subcommittee on Research Animal Care of the University Medical Center.

Animal Preparation, Anesthesia, and Monitoring. Male Sprague-Dawley rats, weighing 240–290 g, were supplied by the University Laboratory Animal Center and were allowed free access to food and water. Animals were anesthetized with 1–2% halothane in 70% N_2O/30% O_2. During surgery, body temperature was maintained at 37.0 ± 0.5°C, which corresponds to brain temperature of 37.2 ± 0.5°C (5), using a thermostatically controlled heating blanket and rectal probe (Harvard Apparatus, South Natick, MA). The right femoral artery was cannulated for measuring arterial blood gases, glucose, hematocrit, and blood pressure.

Experimental Model and Grouping. Focal cerebral ischemia was used by intra-arterial suture occlusion of the proximal right middle cerebral artery for 90 mins as described previously (6, 7). Local cortical cerebral perfusion was serially measured by a laser-Doppler flowmetry (Laserflo BMP2; Vasamedics, St Paul, MN) before and during the middle cerebral artery occlusion, on a brief period (10 mins) of reperfusion and at 40 mins after the onset of reperfusion. The representative ischemic core and penumbral areas in the model were determined based on the rat brain atlas (8) and previous studies on the measurements of cerebral blood flow (7, 9). The local cortical cerebral perfusion data were expressed as a percentage of the baseline values.

Animals were randomly assigned to each treatment protocol. The investigators were blinded to the treatment paradigm. CINN was dissolved in 45% aqueous hydroxypropyl-β-cyclodextrin (Sigma-Aldrich Chemical Co, St Louis, MO) to achieve a final concentration of 1.6%. An optimal dosage of CINN at 80 mg/kg was chosen based on its neuroprotective dose–response studies in a rodent model of stroke (5, 6). Animals were injected intravenously either with CINN (80 mg/kg, n = 19) or vehicle (volume of approximately 1.20–1.45 mL; n = 20) over a period of approximately 5 mins starting from the reperfusion onset. The rats were assigned to one of two follow-up groups for evaluating the electrophysiological outcome and the gray and white matter damage and were euthanized after either 7 or 21 days (day 7 or 21) after the ischemic insult.

Somatosensory-Evoked Potential Recordings. Somatosensory-evoked potential (SSEP) recordings were performed before the insult and at day 7 or 21 postinsult by the method described previously (7, 10). All SSEPs that had recognizable waveforms of the first positive (P1) and the first negative (N1) peaks were used for latency and amplitude measurements.

Animal Euthanasia. Euthanasia was performed by decapitation under anesthesia. The brain was sectioned coronally on a cryostat (HM-500O; Microm International GmbH, Walldorf, Germany) at day 7 or 21 (1, 5, 10). Serial coronal sections of 40 μm, with 1-mm interval from the Bregma anteroposterior +4.22 to −6.78 mm, were mounted on poly-L-lysine-coated slides.

Quantification of Gray Matter Damage. One set of sections was stained with 0.5% cresyl violet. Under light microscopy, the areas of neuronal perikarya displaying typical morphologic features of ischemic damage were delineated (1, 5, 10). Brain infarction were measured using a computerized image analyzer (MCID Elite; Imaging Research Inc, Ontario, Canada), as described previously (1, 5, 6, 10), and were expressed as a percentage of the contralateral hemisphere volume. In addition, individual cortical and subcortical (caudoputaminal and hippocampal) infarct sizes were separately calculated.

Cell Counting for Surviving and Degenerative Neurons. Coronal sections obtained between the bregma anteroposterior −0.22 and −0.78 mm was chosen. Six random and nonoverlapping regions (500 × 400 μm^2) were sampled for the ischemic core (the unstained area), the inner (right side to the margin between the stained and unstained area), and the outer (left side to the margin between the stained and unstained area) boundary zones of the infarct areas at the parietal cortex and the caudoputamen. Cell counts were expressed as the mean number of viable neurons/mm^2. Degenerative neurons were examined by Fluoro-Jade B (Chemicon International Inc, Temecula, CA) staining (11).

Quantification of White Matter Damage. Two sets of sections were processed for immunohistochemistry with SMI-31 (phosphorylated component-H of neurofilaments; Sternberger Monoclonals, Baltimore, MD) and myelin basic protein (Chemicon International Inc) antibodies modified by the method described previously (1, 5, 12). Briefly, monoclonal mouse antibodies against SMI-31 and myelin basic protein diluted 1:1000 and 1:500 in phosphate-buffered saline, respectively, were applied to sections overnight at 4°C. Secondary antibody (biotinylated horse antimouse, 1:100; Vector Laboratories, Burlingame, CA) was applied for 1 hr. The avidin/biotinylated horseradish peroxidase complex (the ABC Elite kit; Vector Laboratories) was then applied for 1 hr, and the sections were allowed to develop chromagen in 3,3′-diaminobenzidine+ nickel solution (Sigma-Aldrich Chemical Co) for 4 mins. Negative controls for each antibody, in which the primary antibody was replaced by preimmune serum, were included, and no immunoreactivity was detected in each protocol.

Images of sections encompassing the striatal injury, corresponding to the Bregma anteroposterior +0.7 mm (rostral), −0.3 mm (medial), and −1.3 mm (caudal), were used for the analysis of white matter damage. The integrity of the phosphorylated component-H of neurofilaments (phosphorylated NF-H) in the ischemic caudoputamen, as assessed by the immunoreactivity for SMI-31, was delineated. The white matter index was calculated by the equation: ipsilateral damaged white matter area/corresponding contralateral, intact white matter area (12). In addition, areas of immunopositive reactions for myelin basic protein in the ischemic hemisphere were calculated and were expressed as a percentage of the contralateral hemisphere volume.

Neurobehavioral Testing and Body Weight Measurements. Body weight measurement was used daily. A battery of sensorimotor tests was conducted before and on a daily basis after reperfusion by two observers unaware of treatment protocol. Briefly, two neurologic grading systems were used: a sensorimotor grading scale modified from previously published methods (5, 7, 13). Accordingly, five categories of motor neurologic findings were scored: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to

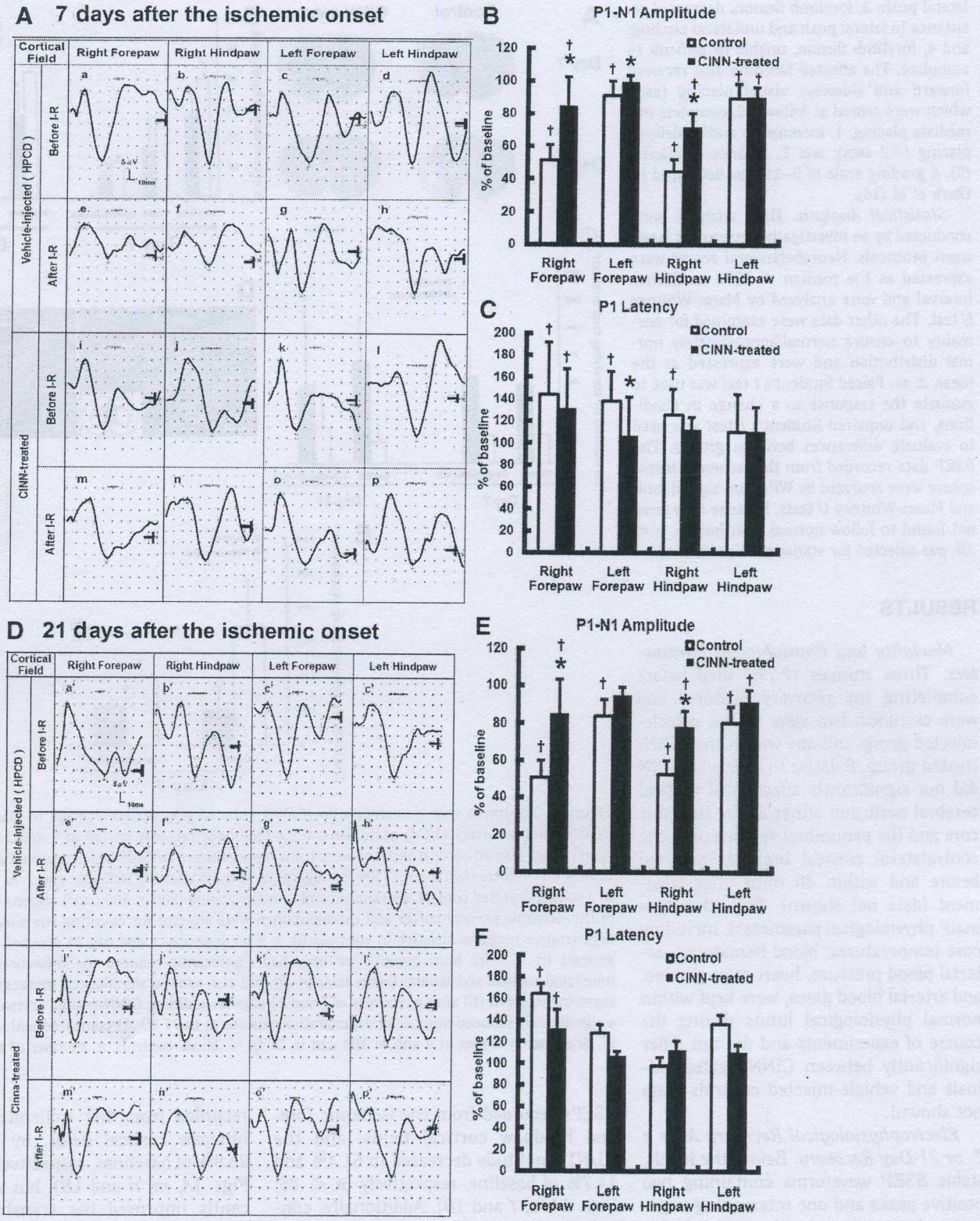

Figure 1. Representative examples of somatosensory-evoked potentials (*SSEPs*) recorded from the right and left SI primary cortices and evoked by contralateral fore- or hindpaw stimulation in controls and cinnamophilin (*CINN*)-treated animals after 7 and 21days after the ischemic insult. Before ischemia–reperfusion, stable SSEP waveforms were recorded (*A*, day 7, *a–d, i–l*; *C*, day 21, *a′–d′, i′–l′*). After cerebral ischemia–reperfusion, the vehicle-injected animals had severely depressant SSEPs recorded from the ischemic fore- and hindpaw cortical fields at day 7 (*A, e, f, B, C*) and day 21 (*D, e′, f′, E, F*) and exhibited transhemispheric diaschisis in the SSEPs recorded at the contralateral fore- and hindpaw cortical fields (*A*, day 7, *g, h, B*, C). CINN not only significantly improved the amplitudes of the SSEPs recorded from both ischemic fore- and hindpaw cortical fields at day 7 (*A, m, n, B*, C) and day 21 (*D, m′, n′, E*, F), respectively, but also diminished transcallosal electrophysiological diaschisis recorded from the contralateral forepaw cortical fields at day 7 (*A, o, B*, C). Scale = 5 μV/10 msec. *$p < .05$ vs. controls and †$p < .05$ vs. baseline values. n = 9 for both the two groups at day 7 and day 21, respectively. *n*, number of animals.

lateral push; 3, forelimb flexion, decreased resistance to lateral push and unilateral circling; and 4, forelimb flexion, unable or difficult to ambulate. The affected forelimb also received forward and sideways visual placing tests, which were scored as follows: 0, complete immediate placing; 1, incomplete and/or delayed placing (<2 secs); and 2, absence of placing (2). A grading scale of 0–28 was developed by Clark et al (14).

Statistical Analysis. Data analyses were conducted by an investigator unaware of treatment protocols. Neurobehavioral scores were expressed as the median ± 95% confidence interval and were analyzed by Mann-Whitney *U* test. The other data were examined for normality to ensure normal/approximately normal distribution and were expressed as the mean ± SD. Paired Student's *t* test was used to evaluate the response to a change in conditions, and unpaired Student's *t* test was used to evaluate differences between groups. The SSEP data recorded from the ischemic hemisphere were analyzed by Wilcoxon signed rank and Mann-Whitney *U* tests, because they were not found to follow normal distribution. $p < .05$ was selected for statistical significance.

RESULTS

Mortality and Physiological Parameters. Three animals (7.7%) died before completing the recovery protocol and were excluded: two were in the vehicle-injected group and one was in the CINN-treated group. Relative to controls, CINN did not significantly affect local cortical cerebral perfusion either at the ischemic core and the penumbral regions or at the contralateral cortical brain as assessed before and within 40 mins after treatment (data not shown). The other animals' physiological parameters, including core temperatures, blood hematocrit, arterial blood pressure, heart rate, glucose, and arterial blood gases, were kept within normal physiological limits during the course of experiments and did not differ significantly between CINN-treated animals and vehicle-injected controls (data not shown).

Electrophysiological Recovery After a 7- or 21-Day Recovery. Before the insult, stable SSEP waveforms containing two positive peaks and one intervening negative peak were consistently recorded after individual fore- and hindpaw stimulation (Figs. 1*A, a–d, i–l* and 1*C, a′–d′, j′–l′*). The amplitude between the first positive (P1) and the first negative (N1) peaks and the P1 latency did not differ significantly between controls and CINN-treated animals ($p > .05$). At day 7, vehicle-injected animals had significantly depressant

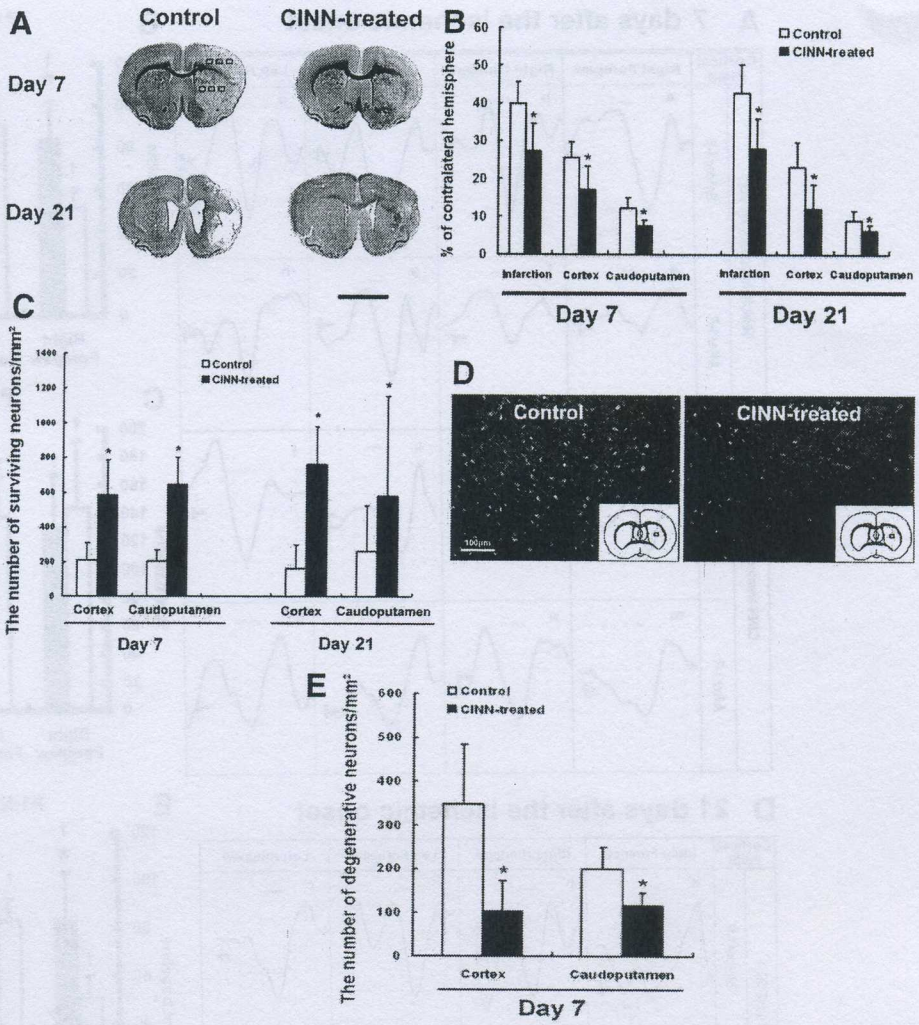

Figure 2. Treatment with cinnamophilin (*CINN*) reduced gray matter damage in rats subjected to middle cerebral artery (*MCA*) occlusion after a prolonged recovery period of 7 and 21 days. *A,* The cresyl violet-stained coronal sections were from representative animals which received an intravenous injection of vehicle (*HPCD*) or CINN (80 mg/kg) at 90 mins after the ischemic onset. As demonstrated in a vehicle-injected control, six random and nonoverlapping (500 × 400 μm^2) regions in the borders of the ischemic parietal cortex and caudoputamen were selected for counting the surviving and the degenerative neurons. Relative to controls (n = 9 for both day 7 and day 21 groups), CINN-treated animals (n = 9 for both groups) not only had significantly reduced the infarction volume and individual cortical and striatal lesion sizes (*A–B*), but also showed significantly increased numbers of surviving neurons (*C*) after a recovery of 7 and 21 days. In addition, CINN-treated animals (n = 9) had a significantly reduced number of degenerative neurons at day 7 (*D–E*) compared with controls (n = 9). Scale bar = 5 mm in *A* and = 100 μm in *D*. *$p < .05$ vs. controls. *n*, number of animals.

SSEPs recorded from the ischemic fore- and hindpaw cortical fields, and the P1/N1 amplitude decreased to 51.4% and 44.7% of baseline, respectively ($p < .05$; Figs. 1*A, e, f* and 1*B*). Additionally, controls had prolonged P1 latencies and decreased P1/N1 amplitudes in the SSEPs recorded postischemia at the contralateral, intact fore- and hindpaw cortical field (transhemispheric electrophysiological diaschisis) relative to baseline values ($p < .05$; Figs. *1A, g, h*; 1*B*; and 1*C*). CINN, however, not only significantly enhanced the P1/N1 amplitude of the SSEPs recorded from the ischemic fore- and hindpaw cortical fields, by 33.2% and 26.9% of baselines, respectively ($p < .05$; Figs. 1*A, m, n* and 1*B*), but also significantly improved the transhemispheric electrophysiological diaschisis recorded from the nonischemic forepaw cortical field ($p < .05$; Figs. 1*A, o*; 1*B*; and 1*C*), compared with vehicle-treated controls.

At day 21, vehicle-injected stroke animals only had modest improvements in the SSEPs recorded from the ischemic hindpaw, but not the ischemic forepaw, cortical fields after cerebral ischemia–

reperfusion over time. The P1/N1 amplitude obtained from the ischemic fore- and hindpaw cortical fields was 50.5% and 52.6% of baseline, respectively, in vehicle-injected controls ($p < .05$; Figs. 1*D, e', f'* and 1*E*). These two groups of animals also had modestly prolonged P1 latency and decreased P1/N1 amplitudes in the SSEPs recorded postischemia at the contralateral, intact fore- and hindpaw cortical field relative to baseline values ($p > .05$; Figs. 1*D, g', h'*; 1*E*; 1*F*). Our results showed that CINN-treated animals had significantly improved P1/N1 amplitude of the SSEPs recorded from the ischemic fore- and hindpaw cortical fields by 34.7% and 25.5% of baseline, respectively, compared with controls ($p < .05$; Figs. 1*D, m', n'* and 1*E*). These CINN-treated animals also showed improved poststroke electrophysiological diaschisis compared with controls (Fig. 1*E–F*). However, the statistical analysis indicated that this difference was not significant ($p > .05$) at day 21.

Gray and White Matter Histology After a 7- or 21-Day Recovery. After a recovery period of 7 days, gray matter damage was reduced by 31.6% in the CINN-treated animals (n = 9; $p < .05$; Figs. 2*A upper panel*, 2*B*), when compared with controls (n = 9). This reflected the CINN-mediated reductions in cortical and subcortical infarct sizes by 33.3% ($p < .05$) and 38.8% ($p < .05$; Fig. 2*B*), respectively. In addition, these CINN-treated animals showed significant increases in the number of the surviving neurons in the penumbral cortex and caudoputamen by 181.2% and 221.9%, respectively ($p < .05$; Fig. 2*C*) and had significant decreases in the number of degenerative neurons at the penumbral cortex and striatum ($p < .05$ and $p < .05$, respectively; Fig. 2*D–E*) as well compared with controls. At day 21, significantly reduced hemispheric, cortical, and subcortical infarct volumes by 34.9% ($p < .05$), 48.1% ($p < .05$), and 32.2% ($p < .05$), respectively (Figs. 2*A lower panel*, B), were observed in the CINN-treated animals (n = 9) compared with controls (n = 9). These CINN-treated animals also had significant increases in the number of surviving neurons in the penumbral cortex and caudoputamen by 366.9% ($p < .05$) and 122.3% ($p < .05$), respectively (Fig. 3*C*) when compared with controls (n = 9). Additionally, CINN treatment significantly improved sensory, motor, and the 28-point neurologic scores ($p < .05$; Fig. 3*A–C*) but did not affect core temperature and body weight (data not shown) taken 1, 3, 7, and 21 days after the ischemic onset compared with control values.

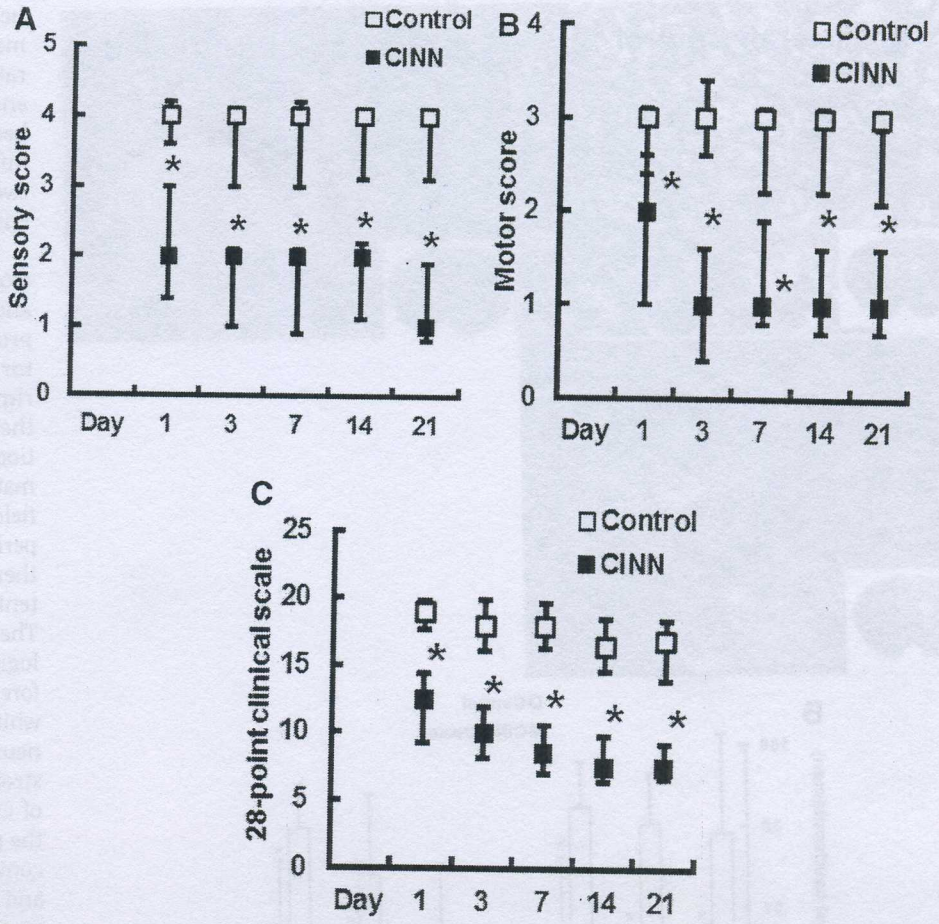

Figure 3. Cinnamophilin (*CINN*) improves neurobehavioral outcomes in rats subjected to middle cerebral artery (*MCA*) occlusion. Relative to time-compatible controls (n = 9, each), CINN-treated animals (n = 9, each) had significantly improved sensorimotor (*A–B*) and 28-point clinical (*C*) scales at days 1, 3, 7, 14, and 21 after ischemia. *$p < .05$ vs. controls; **$p < .01$ vs. controls. *n*, number of animals.

Axonal damage was determined by summation of the areas losing normal axonal bundle structures and the phosphorylated NF-H immunoreactivity within the caudoputamen regions. Regions of damaged axons, sponginess, or destructive morphology in axonal bundles and increased immunoreactivity in the surrounding neuropil were delineated. At day 7, CINN-treated animals showed significant reductions in the axonal damage by 55.5%, 46.3%, and 42.8% in the rostral, medial, and caudal sections, respectively, compared with controls ($p < .05$, respectively; Fig. 4*A–B*). Additionally, CINN decreased the myelin disruption by 28.1% ($p < .05$) in the ischemic hemisphere (Fig. 5*A–B*). Notably, this CINN-mediated axonal and myelin protection remained effective after a recovery period of 21 days. At day 21, CINN significantly reduced axonal damage by 34.0%, 68.6%, and 28.6% in the representative rostral, medial, and caudal sections ($p < .05$, respectively; Fig. 4*B*) and decreased myelin damage by 25.2% in ischemic hemisphere ($p < .05$; Fig. 5*B*) as well.

DISCUSSION

This study demonstrated that administration with CINN effectively reduced gray and white matter damage and improved electrophysiological and neurobehavioral outcomes after transient focal cerebral ischemia in rats. This anatomic, electrophysiological, and functional neuroprotection remained effective up to 21 days after the ischemic insult. Another novel finding was that CINN not only improved the survival of neurons in the penumbral brain, but also decreased neuronal degeneration after cerebral ischemia–reperfusion. Furthermore, we have

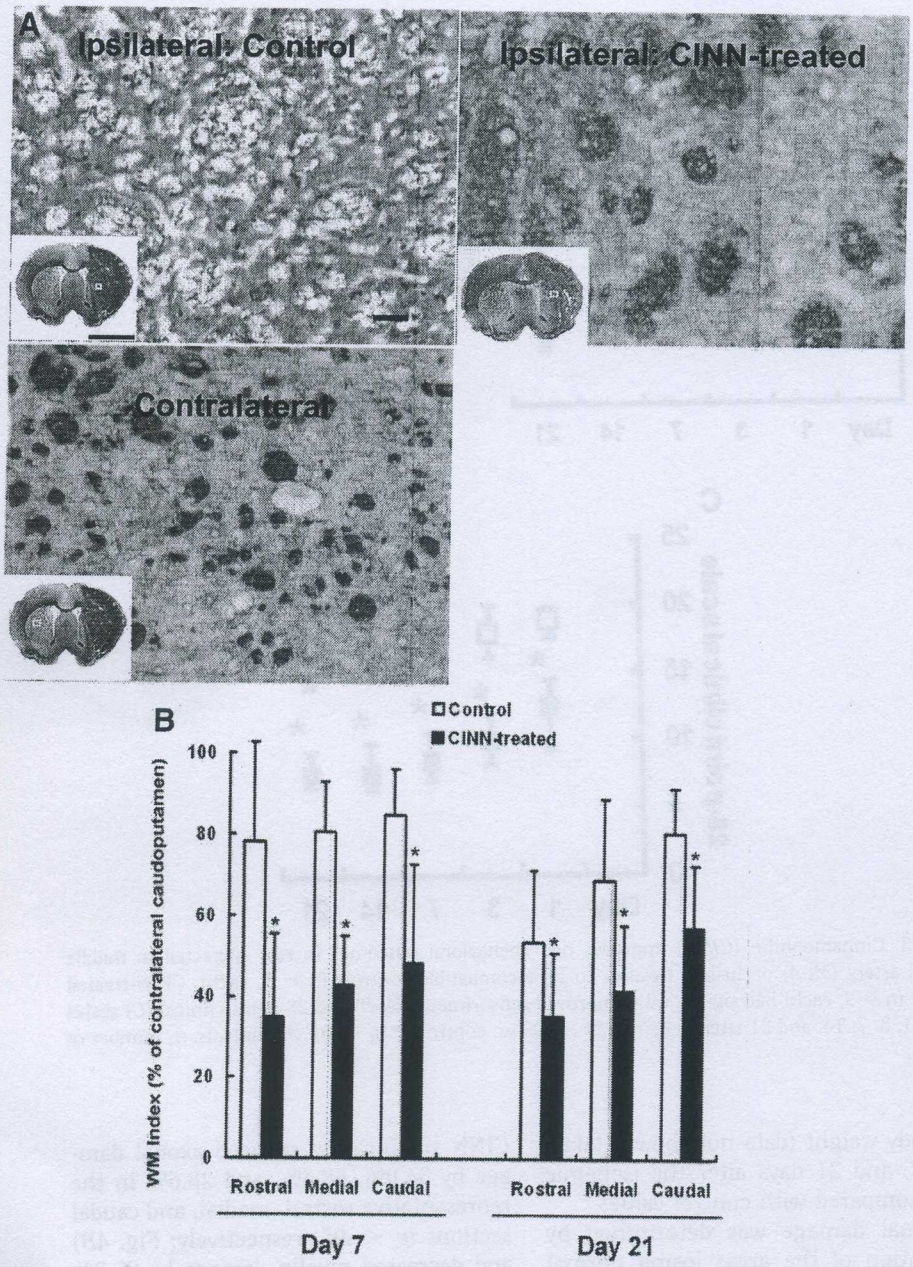

Figure 4. Cinnamophilin (*CINN*) reduced axonal pathology in rats subjected to middle cerebral artery (*MCA*) occlusion. *A*, Representative sections stained with phosphorylated component-H of neurofilaments (phosphorylated NF-H) were obtained from animals after a recovery period of 7 days. Controls (n = 9) had extensive destruction in axonal bundles, as indicated by the loss of phosphorylated NF-H immunohistochemistry, and massive inflammatory cell infiltration (*A, left panel*). In contrast, CINN-treated animals (n = 9) showed modest axonal swelling and minimal cellular infiltration (*A, right panel*). The *bottom panel* in *A* showed the typical phosphorylated NF-H stain in the contralateral, intact brain. After a recovery of 7 and 21 days, (*B*) CINN-treated groups (n = 9 for both groups) had significantly attenuated white matter (*WM*) damage in the representative rostral (the bregma anteroposterior [*AP*] = +0.7 mm), medial (AP = −0.3 mm), and caudal (AP = −1.3 mm) sections compared with controls (n = 9 for both groups). *$p < .05$ vs. controls. Scale bar = 50 μm in *A* and = 5 mm in *insets*. *n*, number of animals.

observed two types of poststroke reductions in the field electrophysiological response in macroscopically intact brain tissues after transient middle cerebral artery occlusion: 1) peri-infarct changes in the penumbral regions; and 2) remote changes in the contralateral hemisphere. Both types of poststroke reductions in field electrophysiological responses, however, could be improved with CINN treatment. This neuroprotection observed cannot be accounted for by changes in hemodilution (as measured by blood hematocrit), arterial blood pressure, heart rate, glucose, core temperature, or differences in local cortical cerebral perfusion, because these parameters were not significantly different when compared between vehicle-injected and CINN-treated animals.

The functional, metabolic, and electrophysiological linkages between gray and white matter are essential for the processing and integration of sensorimotor information (15, 16). Optimal sensorimotor functional recovery after stroke, therefore, requires an anatomic and functional integrity between gray and white matter (1, 15). Marked reductions of the field electrophysiological response in the peri-infarct penumbral region indicate that there were relatively widespread and persistent functional disturbances after stroke. The findings with improved electrophysiological outcome observed with CINN therefore justify its ability to decrease gray and white matter damage and, thus, to improve neurobehavioral recoveries after ischemic stroke. Alternatively, the beneficial effects of CINN to abrogate neurodegeneration in the penumbral brain may be another factor contributing to the improved behavioral and electrophysiological outcomes observed here.

One interesting finding in the present study is that there exists persistent transmission failure in the field potentials notably at least up to 7 days in the contralateral, homotopic regions after transient middle cerebral artery occlusion (16, 17). This contralateral change could not be accounted for by a change in ipsilateral brain edema crossing the midline to affect the SSEPs, because brain edema had much subsided after day 3 of the ischemic onset (7, 10, 18). Curiously, we have previously reported that animals treated with melatonin, a well-known antioxidant and free radical scavenger, also had significantly decreased electrophysiological diaschisis in the contralateral hemisphere in addition to the improved postischemic field SSEPs detected in the penumbral brain ipsilaterally (10). In contrast, treatment with Mg^{2+}, a noncompetitive Ca2+ and N-methyl-D-aspartate blocker, deteriorated the postischemic transcallosal electrophysiological diaschisis, although it substantially improved the field potentials in the penumbral brain ipsilaterally (7). It is, therefore, possible that a variety of mechanisms such as free radical formation, abnormal neurotransmitter storage, uptake

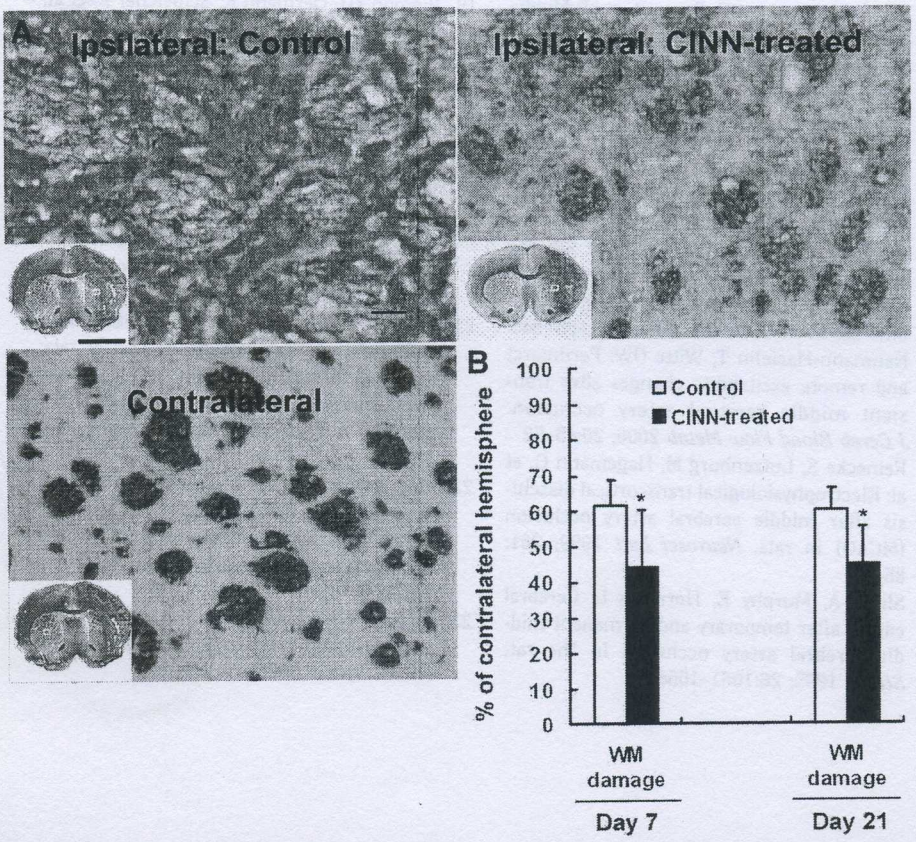

Figure 5. Cinnamophilin (*CINN*) reduced myelin pathology in rats subjected to middle cerebral artery (*MCA*) occlusion. Regions of myelin pathology, as indicated by the myelin basic protein (*MBP*) immunohistochemistry, were delineated. At day 7, CINN-treated animals (n = 9; *A, right panel; B*) had significant attenuation in myelin damage as indicated by the loss of the MBP immunohistochemistry, less extent in the sponginess of the neuropil, and the associated inflammatory cell infiltration relative to controls (n = 9; *A, left panel*). The *lower panel* in *C* demonstrates the normal myelin appearance in the contralateral, intact brain, and the *sampling square* marked in the *inset* indicates the region for illustrations. *B,* The CINN-mediated myelin protection remained effective up to 21 days after the ischemic–reperfusion injury. *$p < .05$ vs. controls. Scale bar = 50 μm in *A* and = 5 mm in *insets*. *n*, number of animals.

and release, or a general stress response may actually underlie the remote excitability changes observed contralaterally after stroke, and this, however, needs further evaluation (16, 19).

Neuroanatomic and electrophysiological plasticity is well known in the hippocampus but remains a subject of some controversy in the contralateral, intact neocortex (16). One of the most probable explanations for the occurrence of contralateral electrophysiological diaschisis is transhemispheric/transcallosal deafferentation (16). This remote excitability changes has also been linked with decreased γ-aminobutyric acid receptor expressions (20) and are widely observed in models of both transient and permanent arterial occlusions (16, 17) as well as in typical cortical lesion and intracerebral hemorrhage models (20–22). In the study, the finding with improved ipsilateral and contralateral field potentials observed with CINN treatment further indicates that CINN may protect both penumbral brain and those brain regions distal from the area of injury against the loss of functional activities after stroke, and, thus, it may facilitate those treated animals to compensate for lost function by using other remote, intact brain areas. Additional studies are, however, needed to clarify the neuroprotective efficacy of CINN in a model of permanent focal cerebral ischemia and also to determine whether the 6-hr therapeutic window, which has been observed previously in the CINN-treated animals after transient focal cerebral ischemia after a 3-day recovery period (6), is applicable to those animals with long-term outcome. Further studies are needed to decipher more molecular events through which CINN leads to functional and electrophysiological neuroprotection observed here. Further studies are also needed to clarify whether CINN could protect against ischemic stoke in reproductively active and senescent female animals, because this experimental cerebral stroke research had been performed exclusively on young male animals so that these results would not be appropriate to generalize to females (23).

In summary, we demonstrate that administering with CINN reduces both gray and white matter pathology and enhances the electrophysiological and neurobehavioral outcomes after cerebral ischemia–reperfusion. Because CINN offers advantage for improving electrophysiological and functional recoveries after stroke, combined with its benefits with a therapeutic window up to 6 hrs and long-lasting neuroprotection without notable adverse effects, it may be worth further investigating in the field of ischemic stroke.

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Experimental studies on ischemic neuroprotection: Criteria for translational significance*

Experimental studies on neuroprotection for ischemic stroke can be broadly categorized into those that address and explore pathophysiological mechanisms of the disease or those that are focused on discovery of potential neuroprotective agents and strategies or a combination thereof. Pharmacologic ischemic protection continues to be a highly desirable goal in the field of stroke. Over the past two decades, numerous experimental studies have focused their attention on neuroprotection with a variety of pharmacologic agents that have demonstrated significant neuroprotection in well-characterized animal models of ischemic stroke. However, translational research in clinical stroke trials with these neuroprotective agents has been disappointing (1). A wide variety of reasons is postulated for the failure of such translational studies; on one end, the validity and appropriate characterization of the animal models used is questioned and an inadequate clinical trial design at the other end of the spectrum. It is imperative that for such laboratory-based studies of translational significance, special attention should be focused on use of well-characterized animal models that incorporate: 1) aged animals reflecting comorbidities (e.g., hypertension, diabetes, and hypercholesterolemia) observed in human stroke as opposed to the use of young and healthy animals; 2) both sexes to allow for sex-based differences; and 3) blinded randomized studies after transient as well as permanent focal ischemia. Additionally, it is important that the route of delivery of neuroprotective agent(s) be translated readily into humans with adequate penetration of the drug through the blood–brain barrier coupled with appropriate dose–response and toxicology studies. Effects of the drug on important physiological parameters (systemic blood pressure, heart rate, arterial blood gases, serum glucose, core body temperature) and on survival as well as long-term functional (at least 30 days) and histologic outcomes must be rigorously evaluated. Furthermore, replication of results in two animal species and two laboratories is desirable. For drugs demonstrating promise in rodent animal models, it is suggested that testing be carried out in primate models to characterize functional outcomes (cognitive, sensorimotor, and behavioral) (2, 3). In fact, over a decade ago, many of these prerequisites and recommendations with subsequent refinements were put forth by a group comprising academic and industry representatives, also known as the Stroke Therapy Academic Industry Roundtable (STAIR) criteria for preclinical translational studies in ischemic stroke (2, 3). It is to be noted that validation and predictors for these criteria for clinical efficacy are lacking in the absence of a successful neuroprotection in a human clinical trial (2, 3).

With this perspective, in this issue of *Critical Care Medicine*, Chen et al (4) report a hypothesis-driven experimental stroke study with cinnamophilin (8R, 8′S)-4, 4′-dihydroxy-3, 3′-dimethoxy-7-oxo-8,8′-neolignan, an agent isolated from *Cinnamomum philippinensis* (5) and significant neuroprotective properties that can be attributed to its pluripotent effects as an antiperoxidative cytoprotectant and free radical scavenger (6) and an anti-inflammatory agent (7). It also exhibits an antiarrhythmic action by inhibiting Ca^{+} (L-type) and Na^{+} currents in cardiac muscle (8, 9). Additionally, the agent readily crosses the blood–brain barrier and has a slow delay in the brain (7). This group of investigators has previously demonstrated that treatment with cinnamophilin provides significant neuroprotection with a prolonged therapeutic window for up to 6 hrs after the onset of focal cerebral ischemia both *in vitro* and *in vivo* (10). In the present study, the authors build on their previous work by a more detailed investigation of longer end points (7 and 21 days) for outcomes in electrophysiological (somatosensory evoked potentials), functional (neurobehavioral), and histologic outcomes by studying the differential effect on gray and white matter (with myelin basic protein immunohistochemistry) in a well-characterized rat model of transient focal ischemia. Few experimental studies in ischemic stroke have focused on differential neuroprotective effects on both gray and white matter as well as on recovery of electrophysiological function. The present study is unique in this regard. Specifically, treatment with cinnamophilin under controlled conditions: 1) improved somatosensory evoked potentials in the ischemic hemisphere as well as the contralateral nonischemic hemisphere at 7 days postinsult as compared with vehicle-treated controls; 2) attenuated cortical and subcortical infarct volumes at day 7 (33% vs. 39% in controls) and at day 21 (48% vs. 32% in controls) and increased survival of degenerating ischemic neurons as well as significantly reduced axonal and myelin injury; 3) resulted in significant improvement in daily sensory and motor functional deficits. It is intriguing to note that the investigators started treatment with cinnamophilin at the onset of reperfusion after 90 mins of focal cerebral ischemia; the present study would have been greatly strengthened if treatment was begun at 6 hrs of reperfusion like in their previous study (10). Despite these limitations, the study by Chen et al provides further evidence for pharmacologic ischemic neuroprotection with cinnamophilin in a number of important domains. Future studies with this agent should incorporate the recommended preclinical STAIR criteria for rigorous experimental investigation that may lead to more meaningful trans-

*See also p. 1130.

Key Words: cinnamophilin; stroke; cerebral ischemia; neuroprotection; translational

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lation into human clinical neuroprotective trials for ischemic stroke (1–3).

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Ten years later, still "gene in a haystack?"*

Differences in human response to illness or injury have been attributed to genetic variation since at least the second century, when familial bleeding disorders were first described (1). Given that genes define, in part, all human phenotypes, it is logical to conclude that variance in DNA sequence alters the host response to illness and injury, and that physiologic stress associated with life-threatening disease exposes genetic anomalies that might otherwise go unnoticed. Thus, the ability to more accurately predict outcomes and to better-understand the link between genetic variance and individual patient responses has obvious appeal to critical care clinicians and researchers alike.

The era of "genomic medicine" has fostered hundreds of studies to assess genetic variation associated with critically ill patient phenotypes. Polymorphisms defining traits of patients with or at risk for sepsis (2) and lung injury (3) are among the most well-reported, and significant numbers of publications describe genetic associations in trauma (4) and burn (5) phenotypes. Pharmacogenomic traits are of particular recent interest, because improved prediction of individual patient responses to medications holds the promise of reducing adverse drug events (6) in the medication-rich environment of critical care. These and numerous other inquires have been conducted to define germline, gene expression, mitochondrial DNA, and epigenetic predictors in critical illness.

In this issue of *Critical Care Medicine*, Dr. Dahmer et al (7) report associations between surfactant protein B gene variants and the need for mechanical ventilation in a study of 395 African American children with community-acquired pneumonia. Two of seven selected tag single nucleotide polymorphisms were significantly associated with mechanical ventilation in univariable analyses, with these same two single nucleotide polymorphisms showing independent, relatively large effects in multivariable regression (odds ratios, 2.27 and 3.00). These effect sizes are notable considering the challenges described and effect sizes typically reported in gene association studies (8). The strengths and limitations of their work are generally well-described by the authors, with a few additional points of note. First, to the researcher's credit, the article largely conforms to published standards for reporting genetic association studies (9). Second, the authors imply that statistical corrections for multiple comparisons are unnecessary given plausible mechanisms underlying the associations studied. Based on this rationale alone, the possibility of a type I error arising from repeated testing should not be dismissed. A more conservative analytical approach would have included correction for multiple comparisons in the present study. Finally, the haplotype analysis should be regarded as preliminary because of the small number of possible haplotypes sufficiently represented and the potential that random effect could account for the statistical significance observed.

Accepting that these and other limitations are outweighed by the strengths of this well-conducted study, what can we glean from the effort? The results are intriguing, in part because of the large effect sizes and plausible mechanisms proposed. As with all gene association studies, these results require independent confirmation because the reproducibility of these types of studies is relatively low (10). If results of the current study are confirmed, then clinical interventions in patients with surfactant protein B risk alleles should be tested. Choosing optimal interventions to study will be challenging because there are diverse preventive, diagnostic, and therapeutic opportunities related to community-acquired pneumonia. Surfactant protein B polymorphisms may be one of the relatively few instances when a small amount of variation in a single gene is sufficient to inform meaningful changes in care.

*See also p. 1138.

Key Words: genetic polymorphisms; candidate gene association; surfactant protein B; surfactant; pneumonia; pediatrics; mechanical ventilation

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