

Cinnamophilin Inhibits Neutrophilic Respiratory Burst and Protects Against Ischemia-Reperfusion Brain Damage

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Abstract

We have shown that administration of Cinnamophilin (CINN) effectively reduced oxidative damage, DNA lipid peroxidation, neutrophil infiltration, and ischemic brain damage by inhibiting oxidative stress and the resulting inflammation in experimental stroke. In this study the potential CINN to ameliorate neutrophilic respiratory burst and reduce neutrophil infiltration was investigated. Neutrophils pretreated or co-treated with CINN, were stimulated by phorbol 12-myristate 13-acetate (PMA) and the levels of superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) produced were determined by dihydroethidium (DHE) and dihydrorhodamine-123 (DHR) fluorescence assays, respectively, while myeloperoxidase activity (MPO) was measured by the guaiacol method. Our results showed that both pretreatment and co-treatment with CINN significantly inhibited H_2O_2 production in PMA-stimulated neutrophils. Additionally, cotreatment, but not pretreatment, with CINN effectively inhibited O_2^- production in the PMA-stimulated neutrophils. Both treatments did not effectively reduce the MPO activity in neutrophil. Finally, animals treated with CINN at reperfusion brain insults significantly reduced brain infarction and neutrophil infiltration, as well as improved neurobehavioral outcome following cerebral ischemic reperfusion. These results support pluripotent neuroprotection actions offered by CINN against cerebral ischemia-reperfusion.

Keywords: Cinnamophilin; Neutrophilic respiratory burst; Cerebral ischemia-reperfusion

Introduction

Up to 40% of stroke patients did not recover their independence [1], which makes stroke a major public issue in the world. There are limited therapeutic treatments, available in clinic, because administration of tissue plasminogen activator (tPA) within 3 hours of symptom onset only benefits a small proportion of stroke patients [2-4], and also increases a risk of hemorrhagic transformation which may potentiate ischemic neuronal damage [5-9]. Thus, there is an urgent need for safe and effective neuroprotective agents that can be beneficial for the majority of stroke patients.

It is well known that neutrophils play an important role in the pathogenesis of ischemia reperfusion injury by releasing a variety of oxygen radicals including O_2^- , H_2O_2 , and OH^- . [10,11].

Following cerebral ischemia-reperfusion, neutrophils produce a variety of reactive oxygen species (ROS) when they are activated during inflammatory responses [12], a process which is known as the neutrophilic respiratory burst. NADPH oxidase which is located in neutrophils catalyzes the generation of O_2^- from oxygen and NADPH [13-15] while O_2^- is converted to H_2O_2 by superoxide dismutase (SOD). Neutrophils utilize myeloperoxidase to further combine H_2O_2 with chlorides to produce hypochlorite. Therefore, neutrophilic respiratory burst results in the release of O_2^- and H_2O_2 , which play an important mechanism of cerebral ischemia-reperfusion injury. It has been shown that neutrophil depletion or inhibition of neutrophil function exerted significant protective effects in ischemia reperfusion-induced brain injury [16-19]. One strategy to protect the brain against ischemic-reperfusion injury is to improve the endogenous antioxidant defense of the tissues at risk, to decrease oxidative damage by scavenging toxic free radicals [20-22] and to inhibit neutrophilic respiratory burst in activated neutrophils [23]. Cinnamophilin, (CINN;

[8R, 8S]-4, 4-dihydroxy-3, 3-dimethoxy-7-oxo-8, 8-neolignan), is a highly lipophilic compound isolated from *Cinnamomum philippinense*, which can readily penetrate the blood-brain barrier with a slow decay in the brain [24,25]. We have previously demonstrated that CINN not only reduced brain infarction and oxidative stress, but also improved behavioral outcome in a mouse model of transient middle cerebral artery (MCA) occlusion [26,27]. More recently, we have demonstrated that CINN exhibited anti-inflammatory actions against ischemic stroke [26]. In the present study, we further examined whether administration of CINN would reduce brain inflammation by inhibiting neutrophilic respiratory burst.

Materials and Methods

Animals

C57BL/6 mice, weighted 19-22 g, and Sprague-Dawley rats, weighed 250-280 g, were supplied by the University Laboratory Animal Center. All procedures performed were approved by the Subcommittee on Research Animal Care of the University Medical Center, and the standards meet the guidelines of the National Institutes of Health.

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Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO) unless otherwise indicated. CINN was isolated from the root of *Cinnamomum philippinense* by the methods previously described [28] and was dissolved in 0.1% dimethylsulfoxide (DMSO, Sigma-Aldrich Co.) for *in vitro* studies.

Transient middle cerebral artery occlusion model

Adult Sprague-Dawley rats were anesthetized with 1% halothane in 70% N₂O/30% O₂. Animals were subjected to intra-arterial filament occlusion of the right proximal middle cerebral artery (MCA) for 90 minutes by the methods previously described [29]. During surgery, animals' rectal temperatures were maintained at 37.0 ± 1.0°C using a thermostatically controlled heating blanket (Harvard Apparatus, South Natick, MA, USA) and heating lamp. Effective MCA occlusion was confirmed by the measurements of a Laser-Doppler flowmetry (LaserFlo BMP2, Vasamedics, St. Paul, MN, USA). Sham-operated rat received similar surgical procedures excluding vascular opening and filament insertion.

Drug administration

Animals were randomly assigned to treatment protocol. The investigators were blinded to the treatment paradigm. CINN was dissolved in 45% aqueous hydroxypropyl-β-cyclodextrin (HPCD). An optimal dosage of CINN at 80 mg/kg was chosen based on its neuroprotective dose-response studies in a rodent model of stroke [27]. Animals were administered intravenously with CINN (80 mg/kg) or the same volume of HPCD at 2 hours after the onset of MCA occlusion.

Neurobehavioral testing

Body weight measurements and neurologic evaluations were assessed at 72 hours after the ischemia-reperfusion insult by one observer unaware of treatment protocol. Two neurologic grading systems were used: (i) a sensorimotor grading scale modified from previously published methods [30] with five categories (0-4) for forward and sideways visual placing tests of the affected forelimb and five categories (0-4) for motor outcome and (ii) a grading scale of 0-28 developed by Clark et al. [31].

Animal sacrifice and quantification of ischemic damage

Following 72 hours of survival, animal was sacrificed under anesthesia by transcardiac perfusion accomplished with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Brains were removed, stored in the same fixative for 24 hours, and sequentially immersed in 15 and 30% sucrose at 4°C for 48 hours. The brains were then embedded in Optimal Cutting Temperature compound (OCT, Miles Inc., Elkhart, IN, USA) and frozen in liquid nitrogen. The brains were sectioned coronally on a cryostat (HM-5000, Microm International GmbH, Walldorf, Germany). Serial sections of 40 μm at eight preselected coronal levels, with 1-mm intervals from the stereotaxic coordinates of the Bregma AP +2.22 to -4.78 mm, were mounted on poly-L-lysine-coated slides and dried at 37°C overnight. Brain infarction was determined by staining preselected brain slices with hematoxylin and eosin stain (HE). Under light microscopy, the areas of neuronal perikarya displaying typical morphological features of ischemic damage were delineated. Infarction volume was measured using a computerized image analyzer (MCID Elite, Imaging Research Inc., Ontario, Canada) and was expressed as a percentage of the contralateral hemisphere volume [29].

Detection of free radicals using dihydroethidium *in situ*

Brain sections were incubated with dihydroethidium (DHE) (stock solution, 1 mM in dimethyl sulfoxide, diluted to 5 μm with PBS, Molecular Probes, Eugene, OR, USA) in a humidified chamber, at 37°C for 30 minutes. The sections were rinsed in PBS for 15 minutes and incubated with DAPI in PBS for 15 seconds in a dark chamber, and then were rinsed in PBS and mounted with Aquamount. Fluorescence was assessed at excitation 365 nm and emission >420 nm for DAPI and was at excitation 510-550 nm and emission >580 nm for ethidium detection. The intensity of the oxidized HET on the ischemic brain was observed and compared between the CINN-treated and vehicle-treated animals.

Immunohistochemistry for myeloperoxidase

Coronal brain sections were cut into 40 μm. A set of brain sections was processed for immunohistochemistry with polyclonal rabbit anti-myeloperoxidase antibody (1:200, Thermo Fisher Scientific, Fremont, CA, USA) at 4°C overnight. After washes with PBS for 5 minutes three times, the bound antibody was visualized by labeling with goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate (FITC) conjugate (Molecular Probes) as a fluorescent probe. Fluorescence was assessed at excitation 488 nm and emission 520 nm for FITC detection.

Neutrophils isolation

Mice were intraperitoneally injected with 5 mg/kg lipopolysaccharide (LPS) to induce neutrophils in blood. Cardiac blood collected 24 hours later was incubated with ACK buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA) for 6 minutes, followed by centrifugation at 800 g to remove red blood cells. Cells were then washed with Hank's balanced salt solution (HBSS, GIBCO, Grand Island, NY, USA) containing 0.05% bovine serum albumin (BSA).

The cytotoxic effect of CINN on neutrophils

For cytotoxicity investigations, neutrophils were pre-incubated with a fixed concentration of CINN (1-300 μm), phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) or vehicle (0.1% DMSO) for 30 minutes. Cytotoxicity was determined by the propidium iodine (PI; 50 μg/mL) staining at 24 hours after treatment.

Respiratory burst assay in neutrophils

PMA (20 ng/ml) was added for 20 minutes to induce the production of H₂O₂ and O₂⁻. Neutrophils were either pretreated or cotreated with CINN at different doses (1-100 μM) for 15 minutes followed by staining with dihydrorhodamine-123 (DHR-123; FL1) to label H₂O₂ or with dihydroethidium (DHE; FL2) to label O₂⁻ at a final concentration of 10 μM for 5 minutes. Neutrophils were gated based on the FSC/SSC scatter on a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using the Cell Quest software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data were presented as the mean ± standard deviation of the mean (S. D.). The distribution of the data was verified by Levene tests before post hoc comparisons. Cytotoxic effects of CINN on the viability of neutrophil were analyzed by one-way analysis of variance (one-way ANOVA) with LSD protected least significant difference post hoc comparison. The data of respiratory burst of neutrophils were analyzed by Kruskal-Wallis/Mann-Whitney U tests because they were not found to follow normal distribution. The other data with normal/

approximately normal distribution were analyzed by using unpaired Student's *t* test. $P < 0.05$ was selected for statistical significance.

Results

CINN reduced brain infarction following MCA occlusion at 3 days

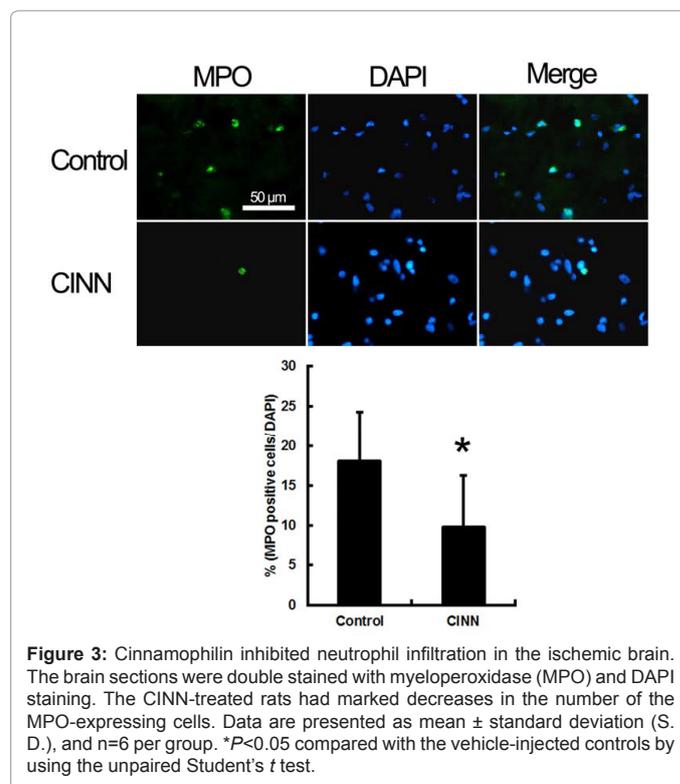
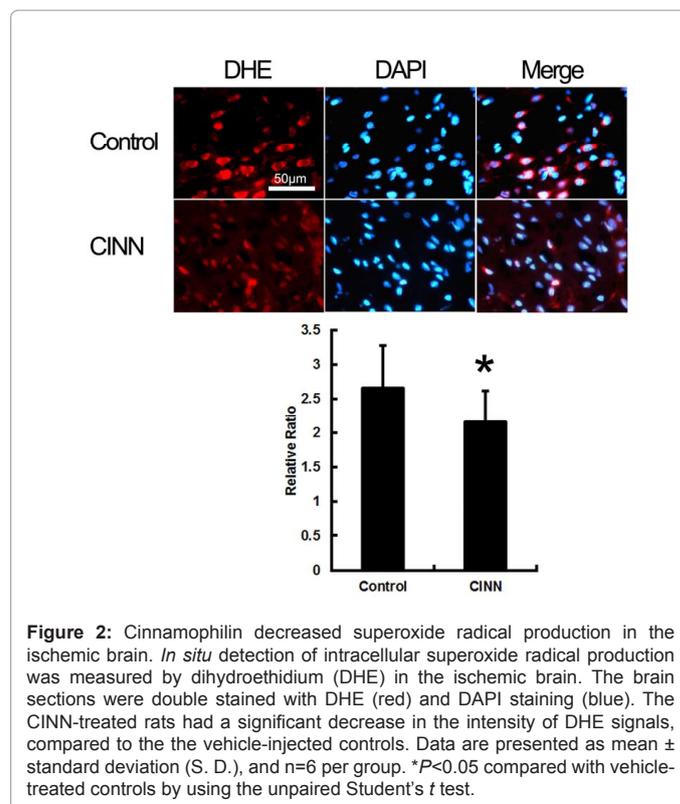
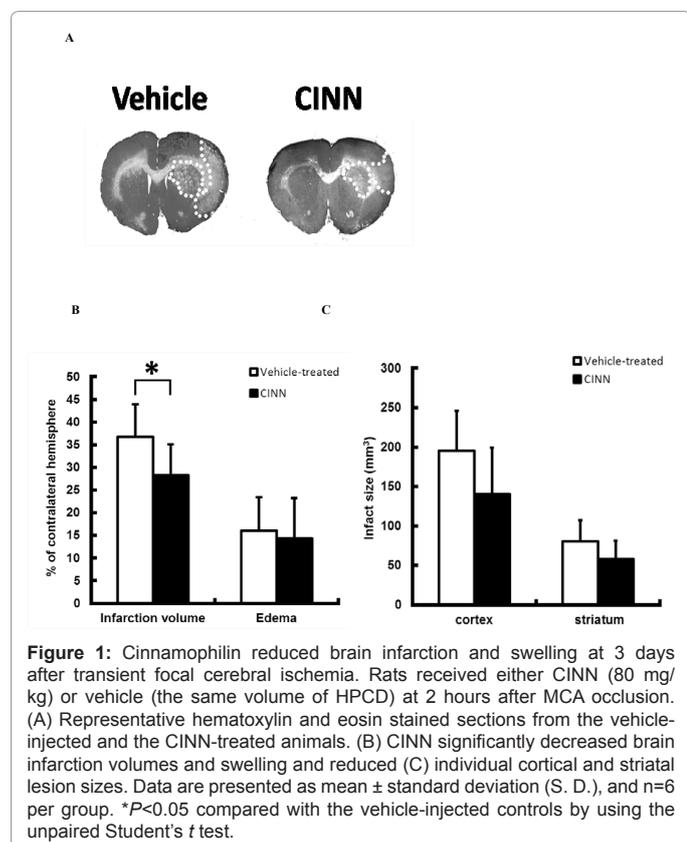
Animal treated either with CINN or vehicle did not have altered local cortical blood perfusion or core temperature during a course of surgery (data not shown). Relative to controls, animals treated with CINN had significant reduced brain infarction and hemispheric edema. Treatment with CINN resulted in 22.9% reduction in infarction volumes ($P < 0.05$; Figure 1A and 1B). However, CINN neither significantly reduced brain swelling nor effectively affected individual cortical and striatal infarcts (Figure 1B and C; $P > .05$, respectively).

CINN inhibited superoxide production and neutrophil infiltration in the brain of animals subjected to transient MCA occlusion

Intracellular production of O_2^- was measured by the staining of DHE. CINN administration significantly decreased DHE signals in the ischemic brain sections (Figure 2, $P < 0.05$). Thus, CINN could attenuate the *in situ* accumulation of O_2^- in the ischemic brain. In vehicle-treated controls, the ischemic reperfusion insult induced dramatically increased numbers of the MPO-expressing cells in the ischemic territory. CINN-treatment animals, however, significantly reduced the number of MPO-immunopositive cells, compared with controls (Figure 3, $P < 0.05$).

Effect of CINN on cell viability of neutrophils

The cell viability was determined by the propidium iodide assay.



No significant cytotoxicity to neutrophils was found with CINN at the concentrations of 1 to 100 μ M (Figure 4). CINN (300 μ M), however, exhibited increased cytotoxicity to neutrophils.

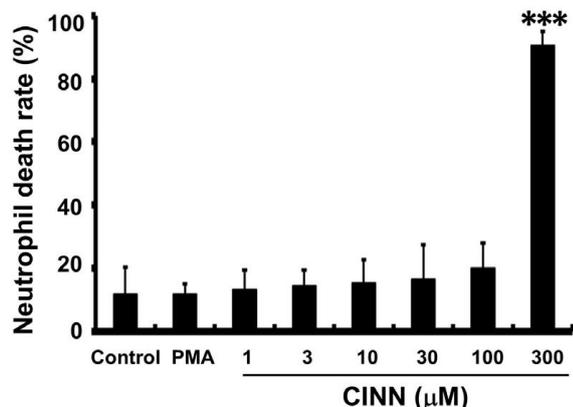


Figure 4: Cytotoxic effect of cinnamophilin on the viability of neutrophil. The cells were treated with PMA (20 ng/ml) or CINN (1 to 300 µM) for 24 hours and the viability of neutrophils were determined by propidium iodide stained. Data are expressed by the mean ± standard deviation (S. D.), and n=3 per group. ****P*<0.001 compared with vehicle-treated controls by using one-way analysis of variance (one-way ANOVA) with LSD protected least significant difference post hoc comparison.

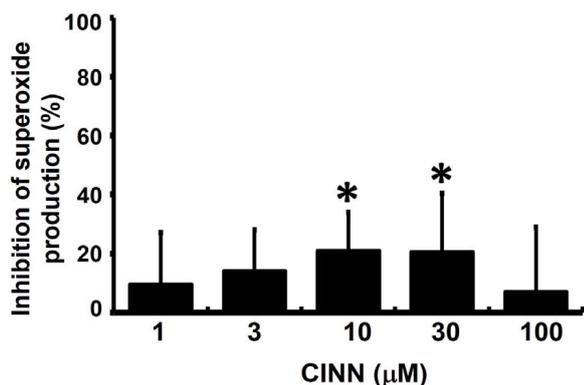


Figure 5: Inhibitory effects of cinnamophilin on superoxide radical production in the PMA-treated neutrophils. The cells were cotreated with CINN (1 to 100 µM) and stimulated with PMA for 15 minutes followed by staining with DHE. The superoxide radical production of cells was determined by flow cytometric assay. Data are expressed by the mean ± standard deviation (S. D.), and n=6 per group. **P*<0.05 compared with controls by using the Kruskal-Wallis/Mann-Whitney *U* tests.

Cotreatment with CINN inhibited superoxide radical production in the PMA-stimulated neutrophils

Pretreatment with CINN at 1-300 µM had no effect on O₂⁻ production of neutrophil after the PMA stimulation (data not shown). Cotreatment with CINN at 10-30 µM effectively reduced the O₂⁻ production by 21% in the PMA-stimulated neutrophils (Figure 5, *P*<0.05).

Pretreatment or cotreatment with CINN inhibited hydrogen peroxide production in neutrophils

Pretreatment with CINN at 3-100 µM dose-dependently inhibited H₂O₂ production by 18-77% in the PMA-stimulated neutrophils (Figure 6A, *P*<0.05). Cotreatment with CINN at 3-100 µM also dose-dependently inhibited H₂O₂ production by 5-88% in

the PMA-stimulated neutrophils (Figure 6B, *P*<0.05). The IC₅₀ for H₂O₂ production was 25.4 µM and 13.9 µM in the pretreatment and cotreatment CINN protocols, respectively. We further investigated the effect of CINN on the MPO activity in the PMA-stimulated neutrophils, and have observed that CINN had no effect on the MPO activity in the PMA-stimulated neutrophils (data not shown).

Discussion

Our results demonstrated that CINN could directly inhibit O₂⁻ and H₂O₂ produced by the PMA-induced respiratory burst in neutrophils. Although CINN did not directly suppress the MPO activity of neutrophils, the agent effectively reduced the MPO activity induced by cerebral ischemia-reperfusion injury observed *in vivo* (Figure 3). Thus, CINN could protect the brain from ischemia-reperfusion-induced injury by inhibiting the respiratory burst actions in activated neutrophils.

We have demonstrated that CINN (80 mg/kg) effectively reduced brain damage induced by cerebral ischemic reperfusion insults in rats [27]. Additionally, the agent effectively improved the numbers of the surviving neurons in the penumbral parietal cortex and caudoputamen [27]. Our previous works have also demonstrated that CINN effectively scavenged free radicals and improved the endogenous antioxidant defense to the tissues at risk of brain infarction. Additionally, CINN had antioxidant effects by reducing the malondialdehyde levels in the ischemic brain tissues and the Fe³⁺-induced lipid peroxidation in rat brain homogenate [26]. Thus, treatment with CINN could result in a marked reduction in postischemic accumulations of ROS and, consequently, attenuated the extent of lipid peroxidation, oxidative

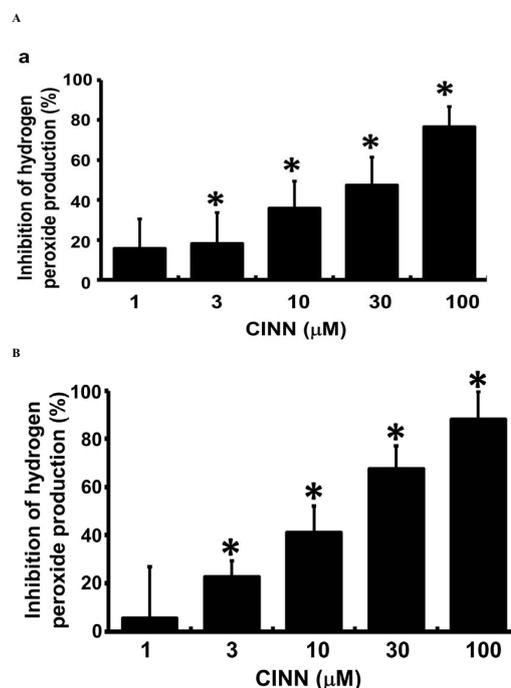


Figure 6: Inhibitory effects of cinnamophilin (CINN) on hydrogen peroxide production in PMA-treated neutrophils. (A) Cells were pretreated with CINN (1 to 100 µM) for 15 minutes, followed by staining with DHR-123. (B) Cells were cotreated with CINN and stimulated with PMA for 15 minutes, followed by staining with DHR-123. The hydrogen peroxide production of cell was determined by flow cytometric assay. Data are expressed by the mean ± standard deviation (S. D.), and n=6 per group. **P*<0.05 compared with vehicle-treated controls by using the Kruskal-Wallis/Mann-Whitney *U* tests.

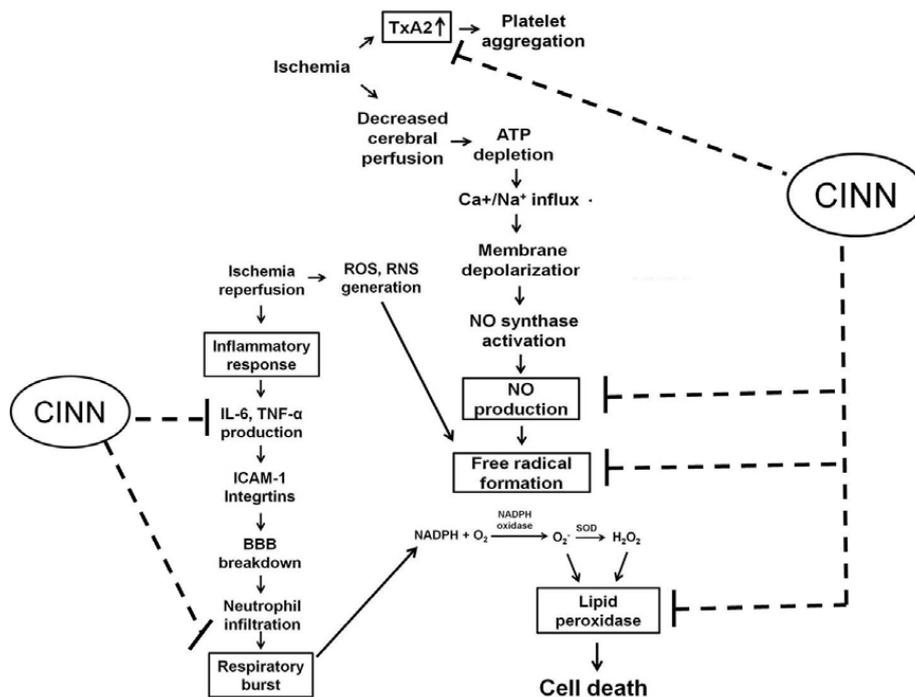


Figure 7: Cinnamophilin provides neuroprotection through pathways that involve inhibition of TXA2, antioxidant, anti-inflammatory effects and anti-respiratory burst.

DNA damage, and the resultant neuronal deaths in the ischemic brain. The present study further demonstrated that CINN effectively reduced neuroinflammation by directly inhibiting neutrophilic respiratory burst in activated neutrophils.

The findings with no effect of CINN against MPO activity in the PMA-activated neutrophils are inconsistent with the *in vivo* data of CINN observed in a rat model of cerebral ischemia-reperfusion. This, however, indicates that molecular mechanisms of neuroprotective effect could be influenced by neuroprotective action in the model of whole animals. Thus, it was very possible that the CINN-mediated reduction in the MPO activity observed *in vivo* might have simply reflected its neuroprotection and anti-inflammatory actions against ischemic stroke. Other mechanisms, in addition to the inhibitory action against the neutrophilic respiratory burst observed with CINN *in vitro*, might also have contributed to the CINN's ability against cerebral ischemia-reperfusion insults observed *in vivo*. Thus, the *in vivo* and *in vitro* studies were essentially independent and might not establish a causal relationship. In addition, the dose-response levels in the present studies actually varied greatly between the *in vivo* and the *in vitro* studies.

Our results confirmed that administration of CINN significantly inhibited the PMA-induced O_2^- and H_2O_2 in neutrophils. However, CINN has no effect to inhibit the MPO activity in neutrophils. Thus, CINN did not directly attenuate the MPO pathway. In previous studies, calphostin C, an inhibitor of protein kinase C (PKC) can obviously reduce the He-Ne laser-induced respiratory burst of neutrophils [32], and the PMA is a stimulator for PKC-mediated NADPH oxidase and nitric oxide (NO) synthase during respiratory burst of neutrophils [33]. Therefore, it was very possible that CINN could also be a PKC inhibitor, and, thus, leading to a decrease in the O_2^- production by reducing the NADPH oxidase activity, and this, however, needs further investigation.

It should be emphasized that fluorescent dyes such as DHR (for H_2O_2) and DHE (for superoxide radical) is not the best method of choice for radical detection, although it is more preferable in the cellular-based studies. The DHR and DHE actually react with many reactive oxygen species (and not only H_2O_2 or superoxide radical), and this non-specific integrity may leads to the production of other interfering substances which could also fluoresce and interfere. Superoxide reacts with DHE and produces a specific reaction product, the 2-hydroxy-ethidium which should be isolated to estimate accurately superoxide radical concentration. This could partly explain the decrease in superoxide radical with the significant high S. D. bar, as seen in Figure 2.

Our previous works have shown that CINN could be served as an effective free radical scavenger and antioxidant, and has multifaceted neuroprotective properties acting against cerebral ischemia-reperfusion injury. In stroke animals, CINN significantly reduced the infarction volumes, proinflammatory cytokine levels, nitric oxide production, and improved neurobehavioral outcomes as well (Figure 7). Therefore, CINN could prevent neuronal damage by its direct antioxidant and anti-inflammatory effects after ischemia-reperfusion. In the present study, we further demonstrated that CINN inhibited neutrophilic respiratory bursts by directly attenuating the PMA-induced O_2^- and H_2O_2 , but not the MPO activity, and thus reduced post-stroke neutrophilic infiltration in the ischemic brain.

Further studies are needed to verify whether CINN would be a PKC inhibitor so as to reduce the NADPH oxidase activity and then to decrease the levels of O_2^- . More studies are also needed to demonstrate the neuroprotective mechanism of CINN in view of its potential role in the field of ischemic stroke.

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