Efficacy of prophylactic flavan-3-ol in permanent focal ischemia in 12-mo-old mice

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1Department of Anesthesiology and Center for Translational Research and Neurodegenerative Disease, College of Medicine, University of Florida, Gainesville, Florida; and 2Departments of Neurology, Psychiatry and Neuroscience, College of Medicine, University of Florida, Gainesville, Florida

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Leonardo CC, Mendes M, Ahmad AS, Doré S. Efficacy of prophylactic flavan-3-ol in permanent focal ischemia in 12-mo-old mice. Am J Physiol Heart Circ Physiol 308: H583–H591, 2015. First published January 9, 2015; doi:10.1152/ajpheart.00239.2014.—The consumption of flavan-3-ol-containing foods, including (−)-epicatechin (EC), has been linked to lower incidence of cardiovascular disease and stroke. We previously demonstrated nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) -dependent EC efficacy in reducing stroke-induced deficits in 2-mo-old mice; yet stroke is primarily a disease of the elderly. Because neuroinflammation, oxidative stress, and vascular dysfunction are hallmarks of aging, we tested whether Nrf2 mediates EC efficacy in aging mice through modulation of glial responses and blood brain barrier permeability. First, we compared anastomosis in naïve wild-type and C57BL/6 Nrf2−/− mice to identify potential differences in cerebrovascular architecture. Data showed no significant differences in the number of anastomoses or mean intersection points, indicating similar gross vascular topology. To assess efficacy and mechanisms of protection, wild-type or Nrf2−/− mice were administered the minimum effective EC dose established in our previous studies before the permanent distal middle cerebral artery occlusion. Similar to previous results with young mice, 12-mo-old wild types also showed significant reductions in infarct volume (41.01 ± 29.57%) and improved performance in removing adhesive tape relative to vehicle-treated controls, whereas a trend toward protection was observed in Nrf2−/−. However, EC did not reduce immunoreactivity for the microglia/macrophage marker anti-ionized calcium-binding adapter molecule 1, suggesting that dampened activation/recruitment did not account for EC protection. Furthermore, there were no differences in mouse IgG extravasation or spontaneous hemorrhage between EC-treated groups. These data demonstrate that EC protection occurs independent of microglia/macrophage modulation or blood brain barrier preservation, suggesting that the glial cell responses in young mice are compensatory to another, and potentially novel, protective mechanism.

The only viable treatment for ischemic stroke, aside from supportive care, is thrombolytic therapy, which must be initiated within 4.5 h of stroke onset (1, 21, 32). In the aging population, however, health risks due to preexisting medical conditions often prohibit this therapeutic option. Because high blood pressure, high cholesterol, diabetes, and other factors limit clinical treatment options in a majority of patients, the ability to mitigate stroke injury through prophylactic neuroprotective “priming” is currently an area of great interest. Complementary and alternative medicinal (CAM) therapy represents a distinct approach whereby the consumption of natural compounds induces protective pathways that enable the brain to mitigate injury when an ischemic event arises. The success of such approaches would therefore benefit patients by eliciting a neuroprotective response, regardless of whether or not they are eligible for clinical treatment after stroke onset.

Our laboratory and others have been investigating the potential of CAM therapies, particularly (−)-epicatechin (EC) and resveratrol, in protective priming against ischemic stroke injury. These natural bioactive compounds are enriched in many food sources, thus representing a safe, noninvasive therapeutic option to bolster endogenous protection against vascular injury and stroke. Indeed, studies have documented improvements in vascular function (13, 52, 56, 60), reduced the risk for cardiovascular disease and stroke (18, 19, 28, 45), and heightened cognition following neurodegenerative conditions (35, 36).

EC and other flavan-3-ols are extensively metabolized in the small intestine and in the liver in humans and rodents following oral consumption (4, 37), which has generated questions as to whether the parent compound or bioactive metabolites are responsible for its beneficial effects. We and others have hypothesized an alternative mechanism of protection involving activation of the nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2). This transcriptional activator regulates cell defense/survival in multiple organ systems throughout the body (25). In response to injury and oxidative and xenobiotic stress, Nrf2 regulates the production of cytoprotective proteins that protect cells from the deleterious microenvironment (31, 64) which persists in various pathological states (39, 40), including stroke (26, 68). Interestingly, the beneficial effects of EC and resveratrol in protecting against vascular dysfunction and cerebral ischemia have been linked to Nrf2 induction (28, 29, 45, 62, 63).

Our laboratory has previously shown that EC effectively reduced infarct volume and basic neurological deficits when measured 72 h after transient brain ischemia (45). Additionally, we have recently documented Nrf2-dependent improvements in anatomical outcomes and complex sensorimotor function in...
young mice after permanent cerebral ischemia (28). However, no studies have evaluated the prophylactic efficacy of EC in aging mice subjected to experimental stroke. Because the aging brain exhibits heightened inflammation and vascular vulnerability, we subjected 12-mo-old mice to permanent cerebral ischemia to ask the question of whether EC can protect the aging brain from permanent focal ischemia and, if so, whether the benefits of EC are associated with glial cell reactivity and cerebrovascular preservation.

MATERIALS AND METHODS

Animal care. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida, in accordance with the Guide for the Care and Use of Laboratory Animals and the guidelines laid down by the National Institutes of Health regarding the care and use of animals for experimental procedures. For all experiments, male wild-type (WT) and Nrf2 knockout (Nrf2<sup>-/-</sup>) C57BL/6 mice were housed in a climate-controlled facility (23 ± 1°C) on a 12-h:12-h reversed light-dark cycle and were provided food and water ad libitum. Mice (4 mo old) were used for anastomosis and hemorrhage evaluations, and 12-mo-old (aging) mice were used for all outcomes measured.

EC treatment and permanent distal middle cerebral artery occlusion. WT or Nrf2<sup>-/-</sup> mice were randomized and administered either methylcellulose vehicle (WT = 8; Nrf2 = 9) or 15 mg/kg of freshly prepared EC (WT = 7; Nrf2 = 9) (Sigma Aldrich, St. Louis, MO) by gavage 90 min before permanent distal middle cerebral artery (MCA) occlusion (pDMCAO), as previously described (28, 45). The pDMCAO procedure was performed under halothane anesthesia (4% induction and 2% maintenance, 1.5/3.0% oxygen/air balance). All possible measures were taken to minimize pain and discomfort, and mice showed no overt signs of stress. Halothane was used because isoflurane has been shown to induce Nrf2-responsive protein expression (42), which would potentially confound the interpretation of results. Complete occlusion was confirmed by severance of the vessel. Because 15 mg/kg was determined to be the minimum effective dose in previous studies using young, healthy mice (28), the same dose was used in the present study for comparative purposes.

Behavioral testing. Because of the relatively mild functional deficits produced by pDMCAO cortical injury, mice were tested for performance on the adhesive removal test and were subjected to a DigiGait (Mouse Specifics, Framingham, MA) gait analysis evaluation.

Adhesive removal test. The adhesive removal task requires forelimb strength, sensation, and complex sensorimotor coordination. Impaired animals mimic clinical pathology in which stroke patients often report weakness and/or loss of function in one or more extremities. For testing, adhesive tape was placed on the planar surface of the forepaw and latency to remove was recorded. Mice were then tested for 3 days, tested for baseline performance before stroke, and posttested 1 day after stroke.

DigiGait. For motor sensory testing using the DigiGait testing, mice were placed on a treadmill, which was set at 20 rpm and activated. Mice were then recorded while running for 3 to 5 s on the treadmill. Paw print placement for each trial was recorded by the DigiGait tracking system. The interface software evaluated various parameters associated with swing and stride. Mice were tested once before stroke and again at 1 day poststroke. For analysis, each parameter was first expressed as a ratio of the opposing side (left/right), and those ratios were expressed relative to baseline gait values.

Histology and immunohistochemistry. Lesion volume and total immunoreactivity were calculated from 16 brain sections collected at even intervals throughout the expanse of the cortical infarct. At 7 days poststroke, mice were perfused, brains were fixed and cryosectioned, and sections were stained with Cresyl violet for lesion volume quantification. Immunohistochemistry was performed to evaluate reactive gliosis by using anti-ionized calcium-binding adapter molecule 1 (Iba1) (1: 1,000; Wako, Richmond, VA) for microglia/macrophages. This antibody is listed and cited in the Journal of Comparative Neurology database. Primary antibody was omitted in control experiments to validate antibody selectivity. Blood brain barrier permeability was assessed using mouse IgG secondary antibody (peroxidase-labeled mouse IgG, 1: 300) (30). For gliosis, biotinylated secondary antibodies were complexed using an elite ABC kit (Vector) and visualized using diaminobenzidine with nickel enhancement (Vector). Images were acquired using an Aperio ScanScope XT, and quantifications were performed using ImageScope software (Aperio, Vista, CA). The ImageScope algorithm “positive pixel count” was used to quantify pixel numbers corresponding to weak positive, positive, and strong positive signal based on preset criteria. Because of the abundance of quiescent microglia/macrophages and resting astrocytes, strong positive pixels were used to identify staining that reflected activated glial cells. The positive pixel counts were expressed as a ratio of the total cortical area measured, and ipsilateral calculations were then expressed relative to the contralateral hemisphere for each section to control for potential variations in antibody penetrance or tissue processing.

Anastomosis. To examine potential variations in the cerebral vasculature of WT and Nrf2<sup>-/-</sup> mice, animals were deeply anesthetized with isoflurane and perfused with PBS followed by 1 ml of black latex paint. Flow rates of both PBS and latex paint did not exceed 1 ml/min. Brains were harvested immediately following perfusion and postfixed in 10% paraformaldehyde for 24 h at 4°C before imaging of the dorsal surface. Images were obtained using a desktop endoscope/ microscope (Oasis Scientific, Greenville, SC). For analysis, the distal branches of the anterior cerebral artery (ACA) and the MCA were traced to the point of anastomosis, which was defined as the narrowest point along the vessel connecting the ACA and MCA (or the midpoint between the nearest branching points in cases of no connecting point). All anastomoses points were then connected to form a line (Fig. 1), and the distance from the midline was determined by measuring the distance from the midline to the anastomosis line. Measurements were taken at 1-mm intervals from the frontal pole to the end of the dorsal surface.

Hemorrhage frequency. For evaluations of rebleeding following stroke, Cresyl violet-stained sections collected throughout the entire cortical infarct were evaluated for intraparenchymal blood. Because of the nature of the occlusion at the cortical surface, traces of surface blood were discounted from the analysis. Only sections showing intraparenchymal blood within or lateral to the cortical infarct were counted, and hemorrhage was recorded as “absent” or “present” for each animal. Hemorrhage frequency was then expressed as the number of mice showing intraparenchymal blood within each experimental group.

Statistical analyses. All analyses were performed blinded such that experimenters performing surgery, behavioral testing, anatomical measures, and data analysis were unaware of the genotypes and treatments. Power analysis was completed based on previous data using the same model. Data are expressed as means ± SE, except in the case of anastomosis, where mean ± SD was used. Statistical significance was set at <i>P</i> < 0.05. Bartlett’s tests showed no significant differences in group variances; therefore, data were evaluated using parametric statistics. Data from experiments evaluating a single factor between two groups were analyzed by two-tailed, unpaired Student’s <i>t</i>-test. For experiments with greater than two groups, significance was determined using one-way ANOVA. Where main effects occurred, Bonferroni post hoc analyses were then used to evaluate multiple group comparisons. For behavioral experiments, the use of a two-by-two factorial design [treatment (vehicle or EC) and time (pretest or posttest)] required analysis by two-way ANOVA, followed by Bonferroni post hoc analyses. The nonparametric Kruskal-Wallis test was used to evaluate hemorrhage frequency. All statistical analyses were
pyknotic nuclei and cellular degeneration in vehicle-treated mice (Fig. 2, A and B), whereas the cortical infarction zones of EC-treated mice showed larger numbers of viable cells and less tissue degradation (Fig. 2, C and D). Quantification showed that EC significantly reduced infarct volume in WT mice relative to vehicle-treated controls ($P = 0.04$), consistent with our previous study in young mice. Of note, EC treatment in 12-mo-old Nrf2$^{-/-}$ mice show a trend toward reductions in infarct volume relative to vehicle-treated Nrf2$^{-/-}$ group; however, this effect did not reach a significant level ($P = 0.12$).

In addition to infarct volume, mice were evaluated for performance on the adhesive removal test, which demonstrates sensitivity in detecting forelimb sensorimotor deficits. The results from WT mice (Fig. 3A) showed a main effect of time, with vehicle-treated mice showing significant elevations in removal time relative to presurgical baseline scores ($P = 0.001$). In contrast, there was no significant difference between pre- and poststroke removal time in EC-treated mice. Furthermore, results from Nrf2$^{-/-}$ mice (Fig. 3B) showed a main effect of time, and subsequent analyses showed significant

Cerebrovascular architecture is similar in WT and Nrf2$^{-/-}$ mice. Anastomosis was evaluated in naïve WT and Nrf2$^{-/-}$ mice to determine whether variations in the cerebral arterial vasculature might account for differences in severity of stroke injury or treatment efficacy. Mice were perfused with black latex paint and the cerebral vasculature was analyzed (Fig. 1) to determine the total number of ACA/MCA intersection points, as well as the mean distances of anastomoses from the dorsal midline. The method for identifying intersection points and line tracing is illustrated in representative macrographs from WT and Nrf2$^{-/-}$ mice (Fig. 1, A and B). Quantification showed no significant differences in the mean intersection distance from the midline or the total number of anastomoses, indicating that cerebral vascular physiology is similar. Data are expressed as mean ± SD.

RESULTS

Fig. 1. Nuclear transcription factor erythroid 2p45-related factor-2 knockout (Nrf2$^{-/-}$) and wild-type (WT) mice show similar cerebrovascular architecture. Anastomosis was assessed to determine potential variations in the cerebral vasculature of WT and Nrf2$^{-/-}$ mice. A and B: representative images from C57BL/6 WT and Nrf2$^{-/-}$ mice show line tracings connecting anastomosis points. C and D: no significant differences were found in the mean intersection distance from the midline or the total number of anastomoses, indicating that cerebral vascular physiology is similar. Data are expressed as mean ± SD.

A power analysis was performed based on previous experiments using the same pdMCAO model and C57BL/6 Nrf2$^{-/-}$ mouse strain. The analysis showed that a sample size of 8 was needed to achieve significant difference [statistical power = 0.80, $\alpha = 0.05$ (2 sided)].

EC improves anatomical and functional outcomes in aging mice. We previously documented the efficacy of EC in young, healthy animals such that a single oral dose of 15 mg/kg 90 min before pdMCAO reduced infarct volume and functional recovery in WT but not in Nrf2$^{-/-}$ mice (28). Therefore, the present study was conducted to determine whether the same prophylactic dose of EC also elicits protection in the aging brains of 12-mo-old mice. Cresyl violet staining revealed expanses of

Fig. 2. (−)-Epicatechin (EC) pretreatment attenuates infarct volume in the aging brain. Representative photomicrographs of Cresyl violet-stained sections from vehicle- and EC-treated WT and Nrf2$^{-/-}$ mice. A and B: tissue voids and regions of degenerating morphology were indistinguishable in vehicle (Veh)-treated WT (N = 8) and Nrf2$^{-/-}$ (N = 9) mice. C and D: sections from EC-treated mice (WT = 7; Nrf2 = 9) displayed more preserved morphology in general, although regions of pyknotic nuclei and cell death were also present. E and F: quantification of infarct volume showed a significant reduction in EC-treated WT mice relative to vehicle controls. EC treatment in Nrf2$^{-/-}$ exhibits a trend toward a decrease in infarction volume; however, this trend did not reach a significant value ($P = 0.12$). Data are expressed as means ± SE. *$P < 0.05$; NS, not significant. Scale bars = 200 μm.

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Elevations in both vehicle-treated (P = 0.002) and EC-treated (P = 0.03) mice relative to their respective baseline scores.

Gait analysis was also evaluated on these mice to determine whether milder deficits could be captured, as the DigiGait system was designed to identify impairments with high sensitivity. Although WT mice showed clear forelimb deficits in removing adhesive tape, no major gait deficits were detected upon analyzing various parameters designed to reflect asymmetries associated with stance/stride length, frequency, etc. (data not shown).

**EC does not alter microglia/macrophage activation 7 days after stroke.** Our previous report in young, healthy mice documented reductions in microglia/macrophage activation and recruitment following EC treatment (28). To determine whether the protective effects of EC in aging mice are associated with alterations in gliosis, sections were subjected to immunohistochemical staining for the microglia/macrophage marker Iba1 (Fig. 4). In sections from vehicle-treated mice, Iba1-positive cells displayed partially to fully activated morphology and were distributed abundantly throughout the cortical infarction zone. Furthermore, there was no observable distinction between the quantity or relative activation state when comparing WT and Nrf2\(^{-/-}\) mice (Fig. 4, A and B). Similarly, sections from EC-treated WT and Nrf2\(^{-/-}\) mice contained regions of abundant Iba1-positive cells throughout the cortical infarct, although EC-treated Nrf2\(^{-/-}\) mice appeared to show less microglia/macrophage recruitment (Fig. 4D) relative to EC-treated WT mice (Fig. 4C). Quantification (Fig. 4E) revealed no significant differences in Iba1 immunoreactivity across treatment (P = 0.17). Astrogliosis was also estimated by quantifying glial fibrillary acidic protein immunoreactivity to determine whether an astrocytic response might be associated with EC protection, and there were no significant differences across treatments or genotypes (data not shown).

**EC does not alter gross vascular permeability or hemorrhage frequency.** Because the protective effects of EC were not associated with alterations in gliosis 7 days after pdMCAO, we

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**Fig. 3.** EC pretreatment promotes functional recovery following stroke. **A:** Vehicle-treated WT mice subjected to permanent distal middle cerebral artery occlusion showed significant elevations in latency to remove adhesive tape relative to presurgical baseline scores, whereas there was no significant difference in removal time for EC-treated WT mice. **B:** latency to remove adhesive tape was significantly elevated in Nrf2\(^{-/-}\) mice after treatment with either vehicle or EC relative to presurgical baseline scores, and there was no significant difference in poststroke removal time between treatments. Data are expressed as means ± SE. *P < 0.05.

**Fig. 4.** EC does not alter microglia/macrophage activation in aging mice. Representative micrographs show immunohistochemical anti-ionized calcium-binding adapter molecule 1 (Iba-1) staining in sections from WT and Nrf2\(^{-/-}\) mice treated with vehicle or EC. **A** and **B:** sections from vehicle-treated WT and Nrf2\(^{-/-}\) mice showed densely distributed cell populations, including large numbers of hypertrophic cells with thick processes that are characteristic of the activated phenotype throughout the ischemic cortex. There was no apparent difference in the number or expanse of activated cells between genotypes. **C** and **D:** cells with activated morphology were numerous throughout the ischemic regions of mice treated with EC, and these cells resembled those in vehicle-treated mice. Treatment with EC appeared to reduce the number of fully activated cells. **E:** quantification revealed no significant differences in Iba1 immunoreactivity between vehicle- and EC-treated WT or Nrf2\(^{-/-}\) mice. IPS/Cont, ratio of ipsilateral to contralateral immunoreactivity. Data are expressed as means ± SE. Scale bars = 200 μm.
sought to determine whether protection might occur through preservation of cerebrovascular integrity. Immunohistochemistry for mouse IgG was performed on sections from WT and Nrf2−/− mice treated with vehicle or EC (Fig. 5). Consistent with the microglia/macrophage profile, animals treated with vehicle (Fig. 5, A and B) showed intense IgG staining throughout the cortical infarction zone that resembled sections from EC-treated mice (Fig. 5, C and D) within each genotype. Quantification of the area occupied by IgG showed no significant differences across treatments ($P = 0.27$) or genotypes ($P = 0.92$).

Despite the finding that there were no significant differences in IgG extravasation into the brain parenchyma, an additional comparison was performed to address whether EC protection could be explained by reductions in spontaneous bleeding after stroke. Because weakening of the cerebral vasculature is a hallmark of the aging process, Cresyl violet-stained sections from 4- and 12-mo-old WT and Nrf2−/− mice were examined to determine whether hemorrhage frequency differed in response to age, treatment, or genotype (Fig. 6). In the 4-mo-old WT mice (young mice group), there was only a single case of intraparenchymal bleeding in the vehicle group, with no events detected in EC-treated animals. In contrast, hemorrhage was detected in four cases with 4-mo-old Nrf2−/− mice, three of which belonged to the EC-treated group. A similar pattern was observed in 12-mo-old mice, with a single case of hemorrhage in a vehicle-treated WT mouse, and four cases in Nrf2−/− mice that were equally distributed across treatments. Despite a trend toward increased hemorrhage in Nrf2−/− mice, there was no significant differences between groups ($P = 0.586$).

**DISCUSSION**

In this study, we tested the hypothesis that prophylactic EC administration would elicit protection from permanent focal ischemia in aging mice and that the benefits of EC would be associated with dampened glial cell reactivity and cerebrovascular preservation. Taken together, data here extend the benefits of prophylactic EC consumption to 12-mo-old mice, thus lending further support for flavan-3-ol use to protect against cerebrovascular disease and stroke. Similar to our previous reports (28, 45), a single prophylactic dose 90 min before pdMCAO effectively reduced infarct volume and reduced behavioral deficits in WT mice. The timing of EC treatment was selected based on peak plasma concentrations previously documented in rodents following oral administration (5, 49), which mirror the timing of stroke onset in the present study. The 15 mg/kg dose represents the minimum effective dose in diminishing microglia/macrophage activation and recruitment, decreasing infarct volume, and improving functional outcomes from our previous report using young mice (28). Consistent with previous data, EC was ineffective in improving histological and behavioral outcomes in aging Nrf2−/− mice, indicating that this transcriptional factor may also partially regulate protection in the aging phenotype.

Although the lack of functional recovery in EC-treated Nrf2−/− mice was pronounced, a trend toward reduced infarct volume in EC-treated Nrf2−/− mice was noted. Based on power analysis from previous experiments using 2-mo-old Nrf2−/− mice, the animal number used was sufficient to achieve adequate statistical power. However, the use of 12-mo-old mice in this study is an important difference that may reflect the need to adjust the animal number, which could not be further extended at this time. Given that these mice did not display an overt aged phenotype either in terms of behavior or anatomical measures, it is likely that the variance arose from deletion of the Nrf2 gene.

Previously, we have shown in young mice that pretreatment with 5, 10, and 15 mg/kg EC resulted in $54.5 \pm 8.3$, $40.1 \pm 14.2\%$, and $50.4 \pm 10.1\%$ reductions in infarction volume, respectively. In the present study, use of 15 mg/kg EC extended its beneficial effect in aging mice and reduced infarct volume by 41.01 $\pm 29.57\%$. Because polyphenolic compounds have been shown to improve outcomes in models of ischemia (7, 27, 29, 50, 58, 59), the goal here was to evaluate possible effects on immune cell activation and cerebrovascular integrity. Interestingly, results did not support the hypothesized mechanisms. Although our previous study showed that the protective effects of EC in young mice were accompanied by...
reductions in microglia/macrophage activation and recruitment, there were no significant differences in these outcome measures in 12-mo-old mice, despite modest qualitative differences. The aging brain is generally considered to display a heightened glial response to insult (8, 34, 54, 65), which may account for the inability of EC to dampen microglia and macrophage activation. However, despite the fact that the aging process is widely assumed to include weakened vascular integrity (10, 16, 23, 24), spontaneous hemorrhage was not significantly altered in any of the experimental groups.

These data beg several questions as to why these outcomes were not altered by treatment, and a few important points may account for these observations. First, underlying differences in vascular permeability and gliosis may not have been captured because of the 7-day time point examined. Additional studies with multiple time points, encompassing both early and late phases of neurodegenerative events, are currently planned. It is also possible that 12-mo-old mice, while considered to be older relative to young mice, have not yet developed the “fully” aged neuropathology that renders them susceptible to heightened glial responses and vascular dysfunction. Importantly, blood brain barrier disruption has been documented in aged (24 mo old) mice (61), supporting the notion that 12-mo-old mice do not yet display the fully aged phenotype. This would explain the lack of associated alterations in IgG extravasation with EC efficacy, and it is an important point to consider when investigating these mechanisms of injury/protection. Therefore, future studies with aged mice (at least 1.5 years) are necessary to fully investigate this possibility, and indeed, several other studies have documented vascular abnormalities in aged rodents (14, 17, 38). Finally, the protective effects of EC may not be contingent on microglia/macrophage responses or blood brain barrier permeability per se. Instead, these outcome measures may simply reflect biological processes that occur in response to other signals within the ischemic microenvironment. Although the nature of such alterations was not uncovered here, these data fill a gap in the literature regarding the mechanisms of flavan-3-ol protection in mice that are transitioning between young and fully aged. Because the neural phenotype changes over time and appears to be species specific to some extent, repeated investigations using multiple ages and animal strains are necessary to validate experimental research (55).

Notably, several key pathways have been identified within the context of CAM therapies. For example, activation of the Nrf2 and pathways by polyphenols has been linked to neuroprotection from oxidative damage (6). In vitro, primary neuronal cultures treated with EC upregulated the expression of the Nrf2-responsive, cytoprotective proteins ferritin light chain, and biliverdin reductase, in addition to heme oxygenase 1. Although we have previously documented the involvement of these pathways in regulating the response to focal ischemia (46, 67), other signaling pathways may mediate the extended benefits observed here. For example, direct antioxidant effect of EC treatment on oxidative stress, as reported in several studies, cannot be ruled out (9, 41, 43), although we do not think it is likely simply on the basis of stoichiometry. It has been proposed that NDAPH oxidase activity augments with aging, thereby it participates in accentuating the oxidative stress and making aging brain more vulnerable to diseases (22, 66). On the other hand, it has been shown that EC inhibits NADPH oxidase in endothelial cells and thereby elevates the level of endothelial nitric oxide and induces vasodilation (53). Moreover, EC may have similar beneficial effects as reported for resveratrol and Gingko biloba, natural compounds that have been extensively studied by our laboratory and others because of its multiplicity of health benefits and pleiotropic actions (44, 46, 57). These natural compounds have been shown to elicit neuroprotection, in part, through downregulation of apoptotic enzymes (2, 20, 44, 48, 51, 57). Additionally, the neuroprotective effects of these natural compounds are also linked with

![Fig. 6. Hemorrhage frequency is not altered by treatment, age, or genotype. Scatter plots show the numbers of young and aging WT and Nrf2−/− mice that displayed intraparenchymal bleeding following administration of vehicle or EC. Despite a trend toward increased hemorrhage for Nrf2−/− mice, analyses revealed no significant differences in intraparenchymal bleeding across treatment, age, or genotype (P = 0.59). E15, 15 mg/kg EC.](http://ajpheart.physiology.org/)

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the reductions in hypoxia-inducible factor-1α mRNA expression (11, 15).

With these emerging lines of evidence, future studies investigating the cell-specific, temporal alterations of these pathways may lead to identification of the key player(s) that mediate protection from delayed degenerative injury following brain ischemia. For example, it is possible that endothelial cell death and subsequent disruption of the neurovascular unit at early time points initiates a series of apoptotic signaling events that lead to delayed neurodegeneration, possibly through hypoxia-inducible factor 1α. Such events are known to occur after cerebral ischemia (3, 12, 33) and would fit with both the injury profile and the effects of EC observed in the present study. Notably, resveratrol has been shown to exert beneficial effects on endothelial cell function, which in turn promotes vascular function (60, 62, 63). Therefore, detailed studies investigating these signaling events throughout the neurodegenerative process will be essential in elucidating the mechanism of action that accounts for EC protection in both the young and aging brain.

Conclusion and potential limitations. Although this study shows the efficacy of prophylactic EC in attenuating permanent focal ischemic brain damage in 12-mo-old mice, there are certain caveats associated with this study. To fully understand and determine the role of EC in regulating ischemic brain damage in aged populations (one of the most common and strong risks factor for stroke), a study on >26-mo old mice is needed. In our young animal studies, we have shown that the beneficial effect of EC is regulated partially through Nrf2 pathways; however, in the current study where we used 12-mo old mice, the use of EC in Nrf2+/− mice shows a trend toward beneficial outcomes in Nrf2−/− mice. This suggests that perhaps with aging, the effect of Nrf2 is weakened and the primary beneficial effect of EC treatment in the aging brain is mediated through regulating the antioxidant defense system. Although WT and Nrf2−/− mice show similar vascular anastomosis system, the vascular protective effect of Nrf2 affecting stroke outcomes cannot be ruled out. To fully explore this avenue, studies with relative and absolute cerebral blood flow changes and vasomotor activity following EC treatment with or without stroke should be performed. To fully understand the role of EC treatment on gliosis on the final outcomes after stroke, the role of microglia should be tested in a time-dependent manner, and constituting of CD68 with Iba-1 could potentially provide further evidence. Nevertheless, it can be safely concluded from our previous studies, along with this current study, that consumption of EC protects the young and aging brain from ischemic brain damage. The exact mechanism(s) pertaining to the beneficial effect of EC is yet to be fully understood.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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23. Kalaria RN. Leonardo CC, Doré S.


