Dehydroascorbic acid, a blood–brain barrier transportable form of vitamin C, mediates potent cerebroprotection in experimental stroke

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Communicated by Samuel J. Danishefsky, Memorial Sloan-Kettering Cancer Center, New York, NY, June 27, 2001 (received for review January 11, 2001)

Neuronal injury in ischemic stroke is partly mediated by cytotoxic reactive oxygen species. Although the antioxidant ascorbic acid (AA) or vitamin C does not penetrate the blood–brain barrier (BBB), its oxidized form, dehydroascorbic acid (DHA), enters the brain by means of facilitative transport. We hypothesized that i.v. DHA would improve outcome after stroke because of its ability to cross the BBB and augment brain antioxidant levels. Reversible or permanent focal cerebral ischemia was created by intraluminal middle cerebral artery occlusion in mice treated with vehicle, AA, or DHA (40, 250, or 500 mg/kg), either before or after ischemia. Given before ischemia, DHA caused dose-dependent increases in postreperfusion cerebral blood flow, with reductions in neurological deficit and mortality. In reperfused cerebral ischemia, mean infarct volume was reduced from 53% and 59% in vehicle- and AA-treated animals, respectively, to 15% in 250 mg/kg DHA-treated animals ($P < 0.05$). Similar significant reductions occurred in nonreperfused cerebral ischemia. Delayed postischemic DHA administration after 15 min or 3 h also mediated improved outcomes. DHA (250 mg/kg or 500 mg/kg) administered at 3 h postischemia reduced infarct volume by 6- to 9-fold, to only 5% with the highest DHA dose ($P < 0.05$). In contrast, AA had no effect on infarct volumes, mortality, or neurological deficits. No differences in the incidence of intracerebral hemorrhage occurred. Unlike exogenous AA, DHA confers *in vivo*, dose-dependent neuroprotection in reperfused and nonreperfused cerebral ischemia at clinically relevant times. As a naturally occurring interconvertible form of AA with BBB permeability, DHA represents a promising pharmacological therapy for stroke based on its effects in this model of cerebral ischemia.

Although stroke is the leading cause of permanent morbidity worldwide (1), current therapy is limited to thrombolysis, which has a narrow therapeutic window and requires sophisticated pretreatment imaging (2, 3). The only Food and Drug Administration-approved therapy for acute ischemic stroke, i.v. recombinant tissue plasminogen activator (rtPA), is indicated for selected patients who can be treated within 3 h of the onset of a stroke. The use of rtPA is associated with an increased risk of intracerebral hemorrhage (ICH) and mortality (4). Largely because of its limitations and risks, rtPA is administered to only a small fraction of all eligible patients (4, 5). The development of other therapies for acute ischemic stroke has been notable in the number of products that have failed to produce a significant advantage over placebo in controlled clinical trials (6). The recurrent clinical failures might be due to heterogeneity in the causes of neural death in humans, drug-associated toxicity at doses required for efficacy, the limited time window required for initiating treatment, or the lack of adequate central nervous system penetration across the blood–brain barrier (BBB). There is still an urgent need for novel therapies that offer efficacy for a broad set of patients.

Acute restoration of blood flow after ischemia leads to the production of reactive oxygen species (7–10), which are directly toxic to neurons and glia, and which may exacerbate leukocyte accumulation (11), microvascular thrombosis, and NO-mediated injury (12–14). Antioxidant strategies have been used successfully to diminish ischemic cerebral tissue damage in animals (15), but the utility of a pharmacological agent as a clinically relevant therapeutic strategy may depend, in part, on its ability to cross the BBB. Although ischemic injury disrupts the integrity of the BBB (16, 17), this disruption is by no means complete (17), and efforts to abrogate oxidant stress in stroke are complicated by the limited ability of antioxidants to cross the BBB (18, 19). Previous work has defined the dehydroascorbic acid (DHA)-GLUT1 transport mechanism by which cells accumulate and retain ascorbic acid (AA) (20–22). Recently, the rapid transport of DHA across the BBB and its retention in the brain as AA was described in rodents (23). DHA has been examined previously as the product of reversible AA oxidation. Early studies demonstrated that DHA is antiscorbutic when given orally, suggesting a metabolic conversion to AA *in vivo* (24). DHA has been given to human volunteers on an experimental basis for the purposes of elucidating its metabolic fate (25, 26). In rodents, effects on salivation and lacrimation were noted after infusion of high doses in unbuffered solutions (27, 28). Because of its unique permeability properties at the BBB, DHA has been re-evaluated for potentially beneficial effects in conditions associated with antioxidant deficiency in the brain.

Here we show that DHA, given i.v. in the setting of murine cerebral ischemia, significantly improves cerebral blood flow and functional outcome and significantly decreases the volume of infarcted brain tissue. The level of cerebroprotection achieved with DHA, not seen with AA, supports our hypothesis that the use of a potent antioxidant precursor, with clearly defined BBB penetrability, has promise in the treatment of thromboembolic stroke in humans.

Materials and Methods

DHA and AA. DHA was purchased from Sigma and was prepared as a 250 mg/ml or 500 mg/ml solution in a sodium acetate/sodium bicarbonate buffer, pH 5.5. AA (250 mg/ml) was prepared in a similar fashion. DHA exists as a dimer in the

Abbreviations: AA, ascorbic acid; BBB, blood–brain barrier; DHA, dehydroascorbic acid; ICH, intracerebral hemorrhage; MCAO, middle cerebral artery occlusion; NS, not significant.

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www.pnas.org/cgi/doi/10.1073/pnas.171325998

crystalline form (29), but it spontaneously converts to a hydrated monomer in aqueous solution (30, 31).

Analyses of crystalline DHA by Fourier transform-IR spectroscopy and proton NMR spectroscopy were consistent with the dimeric structure. Analysis by proton and ^{13}C NMR of DHA in deuterium oxide solution produced results consistent with the hydrated monomer. It may be concluded that DHA was in the monomeric form in the infusion medium and the circulation. The purity of the bulk DHA was determined to be ≈95%, and the AA content was <0.1% by HPLC. The vehicle solution consisted of sodium bicarbonate/sodium acetate buffer prepared without the addition of active agent. Low (40 mg/kg), intermediate (250 mg/kg), and high (500 mg/kg) doses of DHA were used and compared with both intermediate (250 mg/kg) and high (500 mg/kg) doses of AA and vehicle controls.

Murine Model of Cerebral Ischemia. For experiments examining the effect of focal cerebral ischemia on the ability of DHA to cross the BBB and protect cerebral tissue, we used an intraluminal murine model of transient (45 min) or permanent (24 h) right middle cerebral artery occlusion (MCAO) (32). All studies were performed in accordance with an institutionally approved protocol and guidelines provided by the American Academy of Accreditation of Laboratory Animal Care. Normothermic, anesthetized C57BL/6J mice (aged 6–8 weeks and weighing 20–25 g, The Jackson Laboratory) underwent perioperative, bilateral transcranial measurements of cortical cerebral blood flow by using a straight 0.7-mm laser Doppler probe (model PF 303, Perimed, Stockholm) at previously described landmarks (32).

***In Vivo* BBB Transport Studies.** Nine mice were subjected to 2 h of focal ischemia or a sham operation in which the arteriotomy was performed but no occluding suture was placed, and they were immediately killed to assess transport across the BBB of ascorbate (250 mg/kg, $n = 3$), DHA ($n = 3$), and sucrose ($n = 3$), as measured by radiation scintillation counting using dorsal penile vein injections of 5 μCi of ^{14}C-AA (L-[1-^{14}C]-AA, specific activity, 6.6 mCi/mmol, DuPont/NEN), ^{14}C-DHA generated by incubating ^{14}C-AA with ascorbate oxidase (derived from *Cucurbita* species, Sigma), 1 unit/1.0 mmol L-ascorbate, or ^{3}H-sucrose ([fructose-1-^{3}H]sucrose, specific activity 20.0 Ci/mmol, DuPont/NEN), as described (23). The brains were harvested, homogenized in 70% methanol, and prepared for scintillation spectrometry or HPLC. HPLC was performed on the methanol fraction with 1 mmol/liter EDTA added. HPLC samples were separated on a Whatman strong anion exchange Partisil 10 SAX (4.6 × 25 cm) column. A Whatman-type WCS solvent-conditioning column was used, and the eluents were monitored with a Beckman System Gold liquid chromatograph (Beckman Instruments, Irvine, CA) with a diode array detector and radioisotope detector arranged in series. AA was monitored by UV absorbance at 265 nm and by radioactivity. DHA exhibits no absorbance at 265 nm and was monitored by radioactivity only.

Infarct Volume and Neurological Deficits after Transient and Permanent MCAO. To establish the tolerability and efficacy of DHA, initial experimental groups were administered DHA in normal saline immediately before MCAO. Twenty-three hours after either transient (45 min) or permanent occlusion, animals underwent neurological examination using a four-tiered grading system as described (33). Infarct volumes were calculated by digital planimetric analysis of serial 2,3,5-triphenyltetrazolium-stained cerebral sections of 1-mm thickness (Adobe PHOTOSHOP 4.0 and National Institutes of Health IMAGE 1.61), with volumes expressed as the percentage of the ipsilateral hemisphere (32). Mortality was defined as animals that died ahead of schedule. There were 62 animals in the reperfusion cohort (including vehicle, low and intermediate DHA, and intermediate AA cohorts) and 49 animals in the nonreperfused cohort (including vehicle, intermediate DHA, and intermediate AA cohorts).

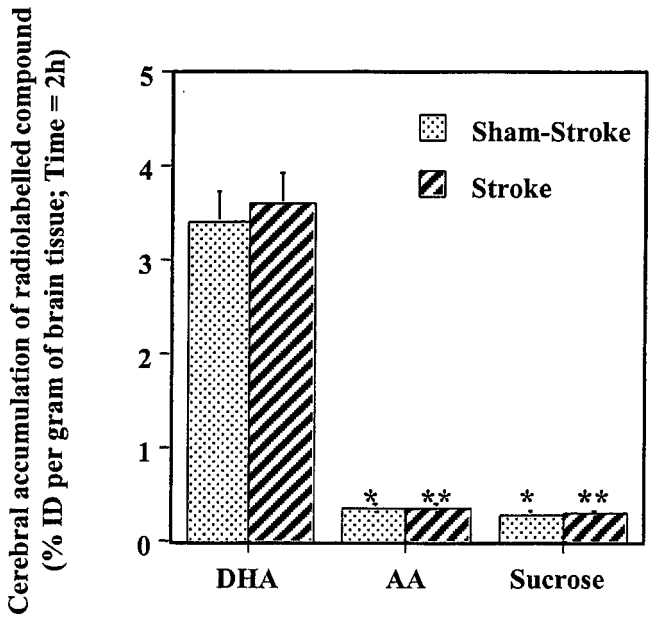

Fig. 1. DHA accumulation. Effects of cerebral ischemia on the accumulation (at 120 min) of DHA, AA, and sucrose in the brain ($n = 3$/cohort). The data are expressed as percentage of injected dose (ID)/g of brain tissue. DHA, but not AA or sucrose, accumulated similarly in cerebral tissue of animals that underwent a sham procedure and in animals that were not subjected to ischemia (*, $P < 0.05$; **, $P < 0.01$).

A separate set of experiments was performed in which an intermediate dose of DHA ($n = 15$) was administered 15 min after permanent vessel occlusion and compared with controls ($n = 11$) or intermediate AA ($n = 13$). Next, the viability of DHA as a delayed therapeutic intervention at 3 h after nonreperfused cerebral ischemia was examined in cohorts receiving vehicle ($n = 11$), high AA ($n = 9$), intermediate DHA ($n = 10$), or high DHA ($n = 8$).

ICH. A previously validated spectrophotometric assay of hemoglobin in brain homogenates was performed to assess the extent of ICH in animals receiving antioxidant treatment after nonreperfused cerebral ischemia (34).

Statistical Analysis. All values are expressed as means ± SEM. Comparisons between means of groups were made with a two-tailed Student's t test for unpaired variables. Differences between groups were considered significant when $P < 0.05$.

Results

DHA Transport Across the Ischemic BBB. Tracer studies have revealed brain accumulation of nearly 4% of the bolus-administered DHA (expressed as percent of injected dose per g of brain tissue) compared with only trace levels of AA and sucrose (a nonmetabolized, nontransportable marker of plasma volume) (23). The induction of cerebral ischemia did not significantly alter the accumulation of radiolabeled compound when compared with sham operation (Fig. 1). In fact, any BBB disruption attendant to this experimental ischemia resulted in slightly decreased AA transit, compared with mild increases for DHA and sucrose [P = not significant (NS) for both]. Previous studies demonstrated that the form of AA, which accumulates in the brains of DHA-injected animals is >85% AA as a result of reduction of transported DHA (23).

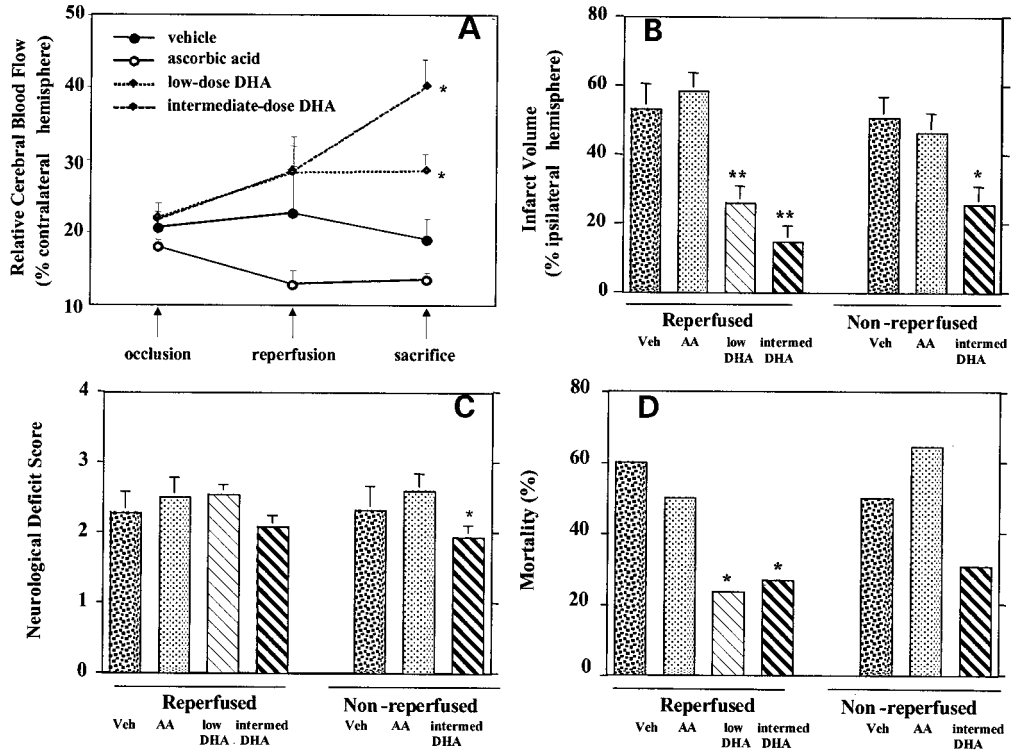

Fig. 2. Effects of low- and intermediate-dose DHA administration before ischemia. Reperfused cerebral ischemia cohort (low dose: $n = 21$; intermediate dose: $n = 16$; AA: $n = 8$; vehicle: $n = 17$). Nonreperfused cerebral ischemia cohort (intermediate dose: $n = 21$; AA: $n = 14$; vehicle: $n = 14$). (*A*) Dose-dependent improvements were demonstrated in cerebral blood flow at the time of death (24 h) after reperfused cerebral ischemia (*, $P < 0.05$ for either DHA doses vs. AA or vehicle). There was a significant increase in cerebral blood flow at death in nonreperfused animals treated with intermediate-dose DHA (*, $P < 0.05$ vs. both AA and vehicle). (*B*) Dose-dependent improvements were demonstrated in infarct volume after reperfused cerebral ischemia (*Left*, **, $P < 0.01$ for both DHA doses vs. AA and vehicle). Improvement was demonstrated in infarct volume after nonreperfused cerebral ischemia (*Right*, *, $P < 0.05$ for intermediate DHA dose vs. vehicle). (*C*) There was a slight decrease in neurological deficit scores after reperfused cerebral ischemia (*Left*) in the intermediate-dose DHA-treated animals compared with AA- and vehicle-treated animals (P = NS). There was a significant decrease in neurological deficit scores after nonreperfused cerebral ischemia (*Right*) in the intermediate-dose DHA-treated animals compared with AA-treated animals ($P < 0.05$). (*D*) There was a significant decrease in mortality after reperfused cerebral ischemia (*Left*) in the low- and intermediate-dose DHA-treated animals compared with vehicle-treated animals ($P < 0.05$). There was a 50% decrease in mortality after nonreperfused cerebral ischemia (*Right*) in the intermediate-dose DHA-treated animals compared with vehicle-treated animals (P = NS).

Effect of Preevent DHA on Outcome After Reperfused and Nonreperfused Cerebral Ischemia. Both intermediate- and low-dose DHA pretreatment of animals undergoing reperfused cerebral ischemia resulted in a dose-dependent improvement in postischemic cerebral blood flow compared with both vehicle- and AA-treated animals ($P < 0.05$ for either DHA group vs. either control) (Fig. 2*A*). In addition, DHA conferred dose-dependent cerebroprotection as evidenced by both decreased infarct volumes ($P < 0.01$ for either DHA vs. either control) (Fig. 2*B Left*) and a small reduction in neurological deficit scores (P = NS) (Fig. 2*C Left*). In contrast, AA treatment was not associated with significant improvements in either infarct size or neurological function, nor did AA treatment reduce overall mortality as was the case with DHA (Fig. 2*D Left*). AA-treated animals died at nearly twice the rate of the DHA-treated cohort ($P < 0.05$ for both DHA vs. vehicle).

A future role for DHA in acute stroke therapy might involve its emergent prehospital administration before any thrombolytic reperfusion. We therefore chose to examine the effect of intermediate-dose DHA on nonreperfused cerebral ischemia as well. As in the previous set of experiments, intermediate-dose DHA improved predeath regional cerebral cortical perfusion whereas AA did not ($P < 0.05$ for DHA vs. both AA and vehicle) (Fig. 2*A*). This improved cerebral blood flow was associated with similar reductions in infarct volume ($P < 0.05$ vs. vehicle) (Fig. 2*B Right*) and death neurological deficit scores ($P < 0.05$ for DHA vs. AA) (Fig. 2*C Right*). As in the setting of reperfusion, DHA reduced mortality by nearly 50% (Fig. 2*D Right*) in the nonreperfused model.

Therapeutic Window of DHA Treatment: Postevent Dosing. When antioxidant treatment was administered 15 min after the ischemic insult, intermediate DHA produced a 6-fold reduction in infarct size ($P < 0.005$) and decreased neurological impairment ($P < 0.005$). Furthermore, cerebroprotection was demonstrated when DHA administration was delayed until 3 h after MCAO. Infarct volume was diminished by 9-fold with high-dose DHA to only $5 \pm 2\%$ ($P < 0.05$ vs. vehicle and AA) (Fig. 3*A*). This finding was associated with diminished neurological deficit ($P < 0.05$) (Fig. 3*B*) and a greater than 2-fold increase in cerebral blood flow at 24 h after injury. Overall mortality was decreased in a dose-dependent manner with DHA treatment (Fig. 3*C*). No significant differences in ICH were detected between cohorts (data not shown). Although cerebroprotection was clearly demonstrated with delayed DHA treatment at 3 h after ischemia, delayed exogenous AA administration conferred no benefit after such injury.

Discussion

Therapeutic, nonenzymatic scavenging of free radicals can be accomplished by AA but only at supraphysiologic concentrations (22). Our results confirm previous findings that i.v. administra-

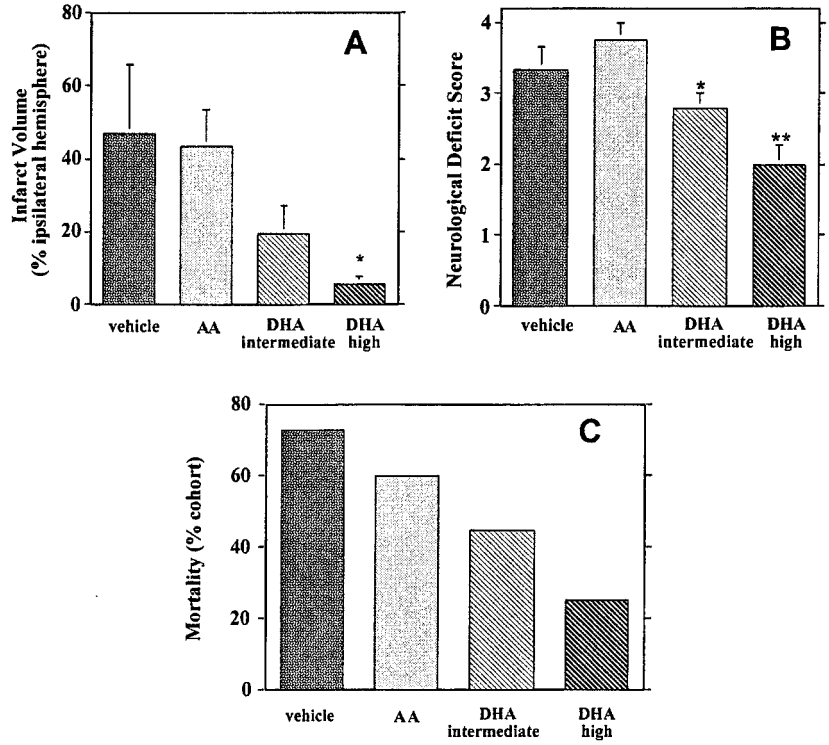

Fig. 3. Delayed intermediate- and high-dose DHA administration. Intermediate-dose DHA ($n = 9$) and high-dose DHA ($n = 8$) were compared with vehicle ($n = 11$) and high-dose AA ($n = 10$) given 3 h after MCAO in nonreperfused cerebral ischemia. (*A*) There was a significant reduction in infarct volume in high-dose DHA animals (*, $P < 0.05$) compared with both AA and vehicle animals. (*B*) There was a significant reduction in neurological deficit scores in intermediate-dose DHA animals (*, $P < 0.05$) and high-dose DHA animals (*, $P < 0.005$) compared with both AA and vehicle animals. (*C*) There was a dose-dependent decrease in mortality in DHA-treated animals compared with AA- and vehicle-treated animals (P = NS).

tion of DHA allows supraphysiologic concentrations of ascorbate to be achieved in the brain whereas AA administration does not (23). Furthermore, these results demonstrate the *in vivo* cerebroprotection conferred by DHA in the setting of both transient and permanent focal cerebral ischemia. In doing so these data further implicate ascorbate in free radical scavenging after cerebral ischemia (27, 28) and underscore the importance of pharmacologically increasing cerebral ascorbate concentrations after cerebral ischemia (35).

The ability of DHA to reduce infarct volume and improve neurological outcomes when administered 3 h after the onset of ischemia suggests that antioxidant depletion is a significant pathophysiologic feature in the brain for at least that duration of time and that it is a suitable target for therapeutic intervention. In the absence of limiting side effects, it is reasonable to hypothesize that increased efficacy could be obtained by administration of higher doses or repeated dosing. Also, the effectiveness of pretreatment of animals with low-dose DHA in the reperfused setting suggests that increasing ascorbate levels in brain may have a protective effect and that efficacy may be the result of an interaction between the timing and level of the dose and onset of infarct.

The concentration of AA in the brain has been reported to be ≈1 mM in humans and rabbits (18, 37, 38) and ≈3 mM in mice and rats (37, 39). AA concentrations might reach as high as 10 mM in the intracellular compartment of brain neurons in rats (37). Thus, with administration of an exogenous antioxidant precursor, it might be necessary to achieve an increase of at least 1 mM in brain AA levels to bring about a pharmacologically meaningful change. An estimate of the degree of elevation achieved in the present study can be calculated. The tracer study indicated that 4% of the administered 5 microCurie dose (SA = 6.6 milliCuries/mM), or 0.030 μM, was taken up by the brain. Assuming that this was 85% converted to AA in the brain (23) and that it was evenly distributed throughout 0.5 ml of brain water, an increase in brain AA concentration of 52 μM would result. The efficacious DHA dose of 250 mg/kg that was found to improve outcome is equivalent to 6.25 mg of drug administered to a 25-g mouse. If one assumed that 4% of this dose were taken up and converted to AA, then the brain would gain 1.2 mM. In a brain water volume of 0.5 ml, this higher dose of DHA therefore would result in an increase in brain AA concentration of 2.4 mM. This might be an overestimate due to nondose-linear uptake (i.e., less than 4% of the dose might enter the brain at the higher dose). Nonetheless, the dose of DHA that produced a significant improvement in cerebral ischemia outcome was theoretically sufficient to increase brain AA levels by a pharmacologically meaningful amount.

Because DHA had a dose-dependent effect on cerebral blood flow, these data also support the contention that local ascorbate may be critical in limiting NO signaling failure mediated by superoxide in the brain microvasculature (22). Ascorbate reacts with superoxide, thus sparing NO, but the reaction of ascorbate with superoxide is 105-fold slower than that of superoxide with superoxide dismutase. Therefore, maintenance of microvascular patency by stabilizing NO-dependent vascular signaling requires very high local levels of ascorbate. Together with previous work this study also suggests that the 10–100 mmol/liter concentrations of ascorbate predicted to inhibit superoxide-dependent vasoconstriction can be achieved with the administration of DHA but not AA (22, 23). The ability of DHA to improve perfusion in ischemic capillary beds subjected to both transient and permanent ischemia has major implications for the treatment of progressive microvascular failure in the setting of clinical stroke.

A major dilemma in the development of potential therapeutic agents in stroke is low efficacy and the high potential for harm when administered to patients, despite previously documented safety and effectiveness in rodent models of cerebral ischemia. These shortcomings have been encountered with various strategies such as a mAb to intercellular adhesion molecule-1, blockade of *N*-methyl-D-aspartate receptors, and an antiplatelet agent (40–43). Even the only currently available treatment of stroke, tissue plasminogen activator, is limited by the risk of devastating ICH when administered after stroke (2, 36). The lack of increased ICH after the delayed administration of DHA after ischemia confers two significant advantages to this agent, which could lead to its utility at clinically relevant time points. As a well-tolerated agent, it could be given safely in the field before arrival in the hospital and the definitive diagnosis of stroke. Furthermore, its low risk of increasing ICH when administered after stroke obviates the expenditure of valuable time within the therapeutic window by eliminating the prerequisite radiographic imaging studies. Taken together, a pharmacological strategy to increase cerebral levels of ascorbate in stroke has tremendous potential to represent the timely translation of basic research into a relevant therapy for thromboembolic stroke in humans.

We thank Dr. Philip Gutin and Daniel Batista for their advice and technical assistance. D.B.A. is supported by CaPCURE and the Eleanor and Paul Stephens Foundation. D.W.G. is a recipient of Grant CA30388 from the National Institutes of Health. D.J.P. is an Established Investigator of the American Heart Association and is supported by National Institutes of Health Grant HL59488. E.S.C. is supported by National Institutes of Health Grant NS02038 and by the William Matheson and Florence and Herbert Irving Foundations. Dr. Samuel J. Danishefsky, the Academy member who communicated this paper, is a member of the Scientific Advisory Board of Progenics Pharmaceuticals, Inc.

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