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Effects of *EGb 761* on Fatty Acid Reincorporation During Reperfusion Following Ischemia in the Brain of the Awake Gerbil

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ABSTRACT

Transient cerebral ischemia (5 min) releases unesterified fatty acids from membrane phospholipids, increasing brain concentrations of fatty acids for up to 1 h following reperfusion. To understand the reported anti-ischemic effect of *Ginkgo biloba* extract (*EGb 761*), we monitored its effect on brain fatty acid reincorporation in a gerbil-stroke model. Both common carotid arteries in awake gerbils were occluded for 5 min, followed by 5 min of reperfusion. Animals were infused intravenously with labeled arachidonic (AA) or palmitic acid (Pam), and rates of incorporation of unlabeled fatty acid from the brain acyl-CoA pool were calculated by the model of Robinson et al. (1992), using quantitative autoradiography and biochemical analysis of brain acyl-CoA. Animals were treated for 14 d with 50 or 150 mg/kg/d *EGb 761* or vehicle. Ischemia-reperfusion had no effect on the rate of unlabeled Pam incorporation into brain phospholipids from palmitoyl-CoA; this rate also was unaffected by *EGb 761*. In contrast, ischemia-reperfusion increased the rate of incorporation of unlabeled AA from brain arachidonoyl-CoA by a factor of 2.3–3.3 compared with the control rate; this factor was further augmented to

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3.6–5.0 by pretreatment with *EGb 761*. There is selective reincorporation of AA compared with Pam into brain phospholipids following ischemia. *EGb 761* further accelerates AA reincorporation, potentially reducing neurotoxic effects of prolonged exposure of brain to high concentrations of AA and its metabolites.

Index Entries: Ischemia; fatty acid metabolism; phospholipid metabolism; *Ginkgo biloba* extract (*EGb 761*); brain; gerbil; arachidonic acid; reincorporation; recovery; damage.

Abbreviations: *EGb 761*, *Ginkgo biloba* extract; [^{3}H]AA, [^{3}H]arachidonic acid; [^{3}H]Pam, [^{3}H]palmitic acid.

INTRODUCTION

Selective neuronal alterations induced by cardio-respiratory arrest in humans can be reproduced using a model of transient global cerebral ischemia in gerbils (Kirino, 1982). This and several other models have shown that a number of metabolic pathways are affected during ischemia or in the early phase of reperfusion, including energy metabolism, cellular ion homeostasis, release of excitatory amino acids, production of oxygen reactive species, and liberation of unesterified fatty acids, lysolipids, and other lipid products from membrane phospholipids (Huang and Sun, 1987; Abe et al., 1992; Halliwell, 1992; Katsura et al., 1993; Deutsch et al., 1996, 1997; Rabin et al., 1997, 1998).

Among fatty acids, the arachidonic acid (AA) that is released initiates a cascade leading to lipoxygenase and cyclooxygenase metabolites, which are augmented during reperfusion following ischemia (Gaudet et al., 1980), and pathology is decreased when their production is decreased. Activation of phospholipase A_2 during ischemia results in increased release of platelet-activating factor, a mediator of inflammation and a putative candidate for activation of immediate early genes involved in delayed neuronal death (Bazan et al., 1991; Doucet and Bazan, 1993). Given the potential pathological role of increased levels of lysolipids and fatty acids during ischemia-reperfusion, acylation of lysolipids with unesterified fatty acids can facilitate recovery by replacing two membrane perturbants with a stable phospholipid and, thus, is crucial to postischemic recovery. No investigation has determined the rate of unesterified fatty acid reincorporation into brain phospholipids, or considered treatment that might enhance this reincorporation during the early reperfusion phase following ischemia.

Ginkgo biloba extract (*EGb 761*) has proven to be effective in various disorders of the central nervous system. A neuroprotective action of *EGb 761* has been documented in animal models of focal or global cerebral ischemia (Le Poucin-Lafitte et al., 1980; Spinnewyn, 1992; Kriegelstein et al., 1995). Several mechanisms have been hypothesized to explain this effect, including affinity of several constituents of *EGb 761* (ginkgolides, bilob-

alide, and flavonoid glycosides) for reactive oxygen species (Pietri et al., 1997), preservation of energy metabolism (Janssens et al., 1995), and modulation of enzymes and metabolites activated or released during ischemia (Bazan and Rodriguez de Turco, 1992). Decrease by *EGb 761* of phospholipase A_2 or C activation, and of the release of platelet-activating factor and AA, could reduce activation of immediate early genes and delayed neuronal death (Bazan and Rodriguez de Turco, 1992). Interestingly, ginkgolide B, a natural and unique constituent of *EGb 761*, is reported to reduce release of free fatty acids and diacylglycerol as well as of platelet-activating factor in ischemia (Panetta et al., 1987).

The purpose of the present study was to investigate the effect of subchronic treatment of *EGb 761* on turnover of free fatty acids in the early phase of postischemic reperfusion in gerbil brain. We used the fatty acid model developed in our laboratory (Robinson et al., 1992) to investigate global cerebral ischemia in awake gerbils. Autoradiography was employed to monitor incorporation of radiolabeled unesterified fatty acids in brain regions affected or not affected by ischemia, and biochemical techniques were used to determine dilution of tracer in the brain acyl-CoA precursor pool and to calculate the rate of reincorporation of unlabeled, unesterified fatty acid into membrane phospholipids, with or without prior *EGb 761* treatment. An abstract presenting partial results of this study has been published (Rabin et al., 1995).

MATERIALS AND METHODS

Materials

[9,10-^{3}H]Palmitic acid ([^{3}H]Pam) (SA, 50 Ci/mmol) and [5,6,8,9,11,12, 14,15-^{3}H]arachidonic acid ([^{3}H]AA) (SA, 185 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Radiochemical purity of both tracers was >98% by thin-layer chromatography. Unesterified fatty acid standards and unlabeled acyl-CoA molecular species were obtained from Sigma (St Louis, MO). Acyl-CoA molecular species were repurified when needed using an oligonucleotide purification cartridge (Applied Biosystems, Foster City, CA) (Deutsch et al., 1994), and purity was checked by HPLC. *G. biloba* extract (*EGb 761*, lot number K 923) was supplied by IPSEN Laboratories (Paris, France).

Experimental Procedures

Animals and Treatment

All experimental procedures were conducted in accordance with the NIH Guidelines for Care and Use of Laboratory Animals (NIH publication 80-23) and were approved by the NICHD Animal Care and Use Committee. Male Mongolian gerbils (*Meriones unguiculatus*) weighing 55–65 g, obtained from Harlan Sprague Dawley (Indianapolis, IN), were

maintained in a temperature-, humidity-, and light-controlled room with free access to standard rodent chow and water. Over a period of 14 d prior to the day of experiment, animals were treated orally using a feeding needle with vehicle, 50 mg/kg, or 150 mg/kg of *G. biloba* extract powder (*EGb 761*) solubilized in water. This dose level was comparable to the dose of 100 mg/kg for 21 d used by Le Poucin-Lafitte et al. (1980). The dose level of 100 mg/kg for 21 d gave a protection against microembolization by microspheres. Blood flow was increased, and ATP decreases were not as great as for untreated animals. In preliminary experiments, we have found that acute or subacute pre- or postischemic treatments with comparable amounts of *EGb 761* were not effective, so chronic treatment was employed and the 100 mg/kg was bracketed by amounts of 50 and 150 mg/kg. Spinnewyn (1992) has shown by histopathological critieria that a dose of 100 mg/kg is neuroprotective and protects against delayed neuronal death in gerbils.

Surgical Preparation

Gerbils were anesthetized with 2–3% halothane (oxygen flow rate, 0.5 mL/min). A surgical thread (5.0 Ethilon, Johnson & Johnson, Somerville, NJ) was looped around both common carotid arteries and was loosely maintained with two silicon rings (Tone et al., 1987). The ends of the suture were led out through the skin on each side of the animal's neck. Sham-operated animals were prepared by simple exposure of common carotid arteries. Two polyethylene catheters (PE 50) containing heparinized physiological saline (100 IU/mL) were implanted in the femoral vein and artery. One percent xylocaine was infiltrated in the femoral and neck incisions before the wounds were closed and the animals were loosely restrained in a plaster cast on a block.

Ischemia-Reperfusion Protocol

During recovery from the surgical procedure, the animals were placed for a minimum of 3 h in a quiet environment with their body temperature maintained at 37 ± 0.5°C and were monitored by a rectal thermometer connected to a heating device. Ischemia was induced in awake animals by pulling the suture ends for 5 min to occlude both common carotid arteries. Blood perfusion to the forebrain was restored for 5 min by removing the suture. Sham-operated animals remained in a controlled environment until tracer infusion. At the end of the ischemic phase, gerbils were divided into different groups according to the duration of reperfusion and type of biochemical analysis conducted.

Tracer Infusion

[^{3}H]Pam and [^{3}H]AA were dried under nitrogen and resuspended in HCO_3-buffered physiological saline containing 3% (wt/vol) "fatty acid-free" bovine serum albumin (Sigma). Tracer solution was pumped into a plastic syringe and connected to the venous catheter via a 23-gage needle.

For autoradiography studies, either [^{3}H]Pam (6.2 mCi/kg) or [^{3}H]AA (170 μCi/kg) (1 mCi/mL for infusion) was infused immediately following

the 5-min ischemic event, at a constant rate of 80 μL/min over a period of 5 min to pulse-label the brain. At the end of infusion of radiolabel, the radioactivity in blood will decrease rapidly to baseline with $t_{1/2} < 0.8$ min (Robinson et al., 1992). We have described a fatty acid model with two compartments, the rapidly turning over precursor pool (i.e., acyl-CoA) ($t_{1/2} \sim 5$–6 s) and the stable compartment of phospholipids in which the fatty acids turn over more slowly (Robinson et al., 1992). Therefore, the radiolabel fatty acid incorporated into the brain phospholipid pool is trapped for the duration of the experiment and is preserved for autoradiographic analysis. Recirculation was continued for 15 min after the radiolabel infusion to allow tracer to disappear completely from the vascular compartment.

To determine dynamic incorporation parameters (i.e., λ), [^{3}H]Pam and [^{3}H]AA (1 mCi/mL) were infused into the femoral vein with a computer-controlled variable-rate infusion pump (Harvard 22, Harvard Apparatus, South Natick, MA) programmed with the following equation: infusion rate = $0.07\ (1 - \exp^{-1.9t})$ where t is infusion time in min. Infusion parameters were set to maintain a constant plasma level of unesterified [^{3}H]Pam or [^{3}H]AA from 0.5 to 5 min. We have shown previously (Washizaki et al., 1994) that tracer specific activities in the plasma unesterified fatty acid and brain acyl-CoA pool are at steady state within 2 min. Acyl-CoA must be sampled immediately because the $t_{1/2}$ is so short. The specific activity at steady-state is required for determination of λ. Just before the start and end of the infusion, blood samples were collected from the femoral artery to determine plasma concentrations of unesterified fatty acids. Blood samples were taken at 1.0, 3.0, and 5.0 min for determination of plasma radioactivity.

At the end of the reperfusion, gerbils were anesthetized by intra-arterial injection of sodium pentobarbital (50 mg) before decapitation, and the brain was rapidly frozen in methylbutane cooled to −65°C for autoradiographic studies, or before microwaving the head by focused-beam irradiation (Cober Electronics, Norwalk, CT) to study dynamic incorporation parameters (Washizaki et al., 1994; Grange et al., 1995).

Autoradiographic Analysis

Twenty-micrometer-thick coronal sections of brain were cut at −20°C and dried on a hot plate before being exposed together with radioactive standards for 10–12 wk to sensitive film (Hyperfilm-3H, Amersham, Arlington Heights, IL). Adjacent sections were kept for staining with cresyl violet for light histological analysis of the extent of ischemic cellular damage. Eleven regions in the forebrain and nine regions unaffected by ischemia in the cerebellum (reference regions; indicated in Table 1 as regions under posterior circulation) (Tone et al., 1987) were selected to determine the normalized uptake of [^{3}H]Pam or [^{3}H]AA (*see* Eq. 2) using an image analyzer program (NIH Image 1.57).

Biochemical Analyses

1. Acyl-CoA analysis: After microwaving, the forebrain was weighed and sonicated with 10 μg of myristoyl-CoA and 10 μg

Table 1
Normalized Brain Incorporation Coefficient $k^*_{norm,br}$ With or Without EGb 761 Treatment Following 5 min of Transient Global Ischemia in Awake Gerbils[a]

		^{3}H-Palmitate			^{3}H-Arachidonate			
		Ischemia-reperfusion dose/treatment			Ischemia-reperfusion dose/treatment			
Brain regions	Control[b] (10)[c]	0 mg/kg/d (8)	50 mg/kg/d (5)	150 mg/kg/d (5)	Control (10)	0 mg/kg/d (5)	50 mg/kg/d (4)[b]	150 mg/kg/d (4)
Anterior Circulation								
Frontoparietal cortex motor	121 ± 3	76 ± 6**	85 ± 4**	109 ± 6	111 ± 3	54 ± 6**	60 ± 3**	54 ± 3**
Somatosensory cortex	128 ± 4	78 ± 5**	90 ± 4**	123 ± 10	116 ± 3	59 ± 7**	68 ± 5**	60 ± 3**
Primary olfactory cortex	101 ± 3	71 ± 6**	74 ± 6**	92 ± 9	91 ± 2	42 ± 6**	62 ± 4**	49 ± 2**
Hippocampus								
CA1	96 ± 2	67 ± 6**	76 ± 1*	88 ± 8	94 ± 2	50 ± 7**	56 ± 3**	47 ± 3**
CA2	94 ± 2	68 ± 6**	77 ± 1*	83 ± 5	95 ± 2	49 ± 6**	58 ± 4**	49 ± 2**
CA3	97 ± 2	68 ± 5**	77 ± 1**	87 ± 5	94 ± 2	50 ± 6**	63 ± 5**	52 ± 2**
CA4	104 ± 2	72 ± 5**	82 ± 2**	92 ± 5	99 ± 2	52 ± 7**	64 ± 4**	53 ± 2**
Dentate gyrus	110 ± 2	77 ± 5**	91 ± 3**	101 ± 6	103 ± 2	59 ± 6**	68 ± 5**	57 ± 2**
Internal capsule	77 ± 3	48 ± 6**	53 ± 5**	64 ± 3	65 ± 4	36 ± 7**	49 ± 3	41 ± 5**
Thalam. paravent. nucl.	116 ± 3	89 ± 6**	99 ± 2*	112 ± 6	115 ± 3	83 ± 7**	80 ± 5**	78 ± 3**
Ventropost. thalam. nucl.	113 ± 4	76 ± 5**	93 ± 2*	104 ± 7	116 ± 4	68 ± 7**	74 ± 6**	67 ± 1**
Post-thalam. nucl. group	114 ± 4	80 ± 4**	87 ± 2**	103 ± 9	113 ± 3	67 ± 7**	73 ± 6**	63 ± 2**
Dorsolateral hypothalam. nucl.	103 ± 2	82 ± 5**	88 ± 3*	104 ± 3	106 ± 3	69 ± 6**	72 ± 5**	70 ± 3**
Basolat-basomed. amygdala	100 ± 2	71 ± 6**	75 ± 4**	90 ± 7	95 ± 2	47 ± 6**	63 ± 4**	54 ± 2**
Posterior Circulation								
Vermis anterior	123 ± 3	144 ± 5**	138 ± 4*	124 ± 3	117 ± 1	118 ± 1	121 ± 4	116 ± 4
Lateral vestibular nucl.	107 ± 1	104 ± 2	111 ± 3	111 ± 2	114 ± 1	109 ± 1	111 ± 3	133 ± 4
Median vestibular nucl.	95 ± 1	94 ± 2	99 ± 3	95 ± 2	104 ± 1	103 ± 2	104 ± 3	105 ± 1
Spinal tract N trigem. nerve	99 ± 2	97 ± 3	102 ± 1	97 ± 1	107 ± 1	109 ± 2	103 ± 2	107 ± 3
Spinal tract trigem. nerve	63 ± 2	62 ± 5	56 ± 5	66 ± 4	59 ± 5	68 ± 3	66 ± 4	65 ± 3
Olive inferior	102 ± 1	99 ± 3	109 ± 4	106 ± 3	114 ± 1	108 ± 3	119 ± 3	111 ± 2
Cochlear nucl. ventral	118 ± 2	124 ± 6	119 ± 5	119 ± 1	121 ± 3	117 ± 3	118 ± 1	116 ± 2
Cereb. gray matter	124 ± 3	126 ± 3	125 ± 3	129 ± 2	115 ± 1	112 ± 1	108 ± 3	112 ± 3
Cereb. white matter	61 ± 3	67 ± 6	56 ± 4	69 ± 3	65 ± 3	74 ± 3	67 ± 3	72 ± 2

[a]Statistical analysis by ANOVA followed by Bonferroni's multiple comparisons test. (Program InStat 2.03). Regions of cerebellum supplied by the posterior circulation were used to define the value $Avg[c^*_{ref}]$ in Eq. 1. Compare between control group and ischemic groups. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

[b]Results from treated and untreated control animals were not statistically different and so were combined.

[c]Number of animals.

heptadecanoyl-CoA in 2 mL KH_2PO_4 (25 m*M*, pH 4.5) for 20 s at 4°C. Next, 2 mL isopropanol were added, and the sample sonicated again for 20 s. The cerebral acyl-CoA was purified by solid-phase extraction on an oligonucleotide purification cartridge (Applied Biosystems, Foster City, CA) (Deutsch et al., 1994, 1996, 1997). The different acyl-CoA molecular species were resolved by HPLC on a Symmetry C-18 column (Waters Millipore Corp., Milford, MA) using a binary gradient of KH_2PO_4 buffer (75 m*M*) and acetonitrile (Woldegiorgis et al., 1985; Deutsch et al. 1996, 1997; Rabin et al., 1997, 1998). Absorbance was measured at 260 nm with a Gilson UV spectrophotometer. The fractions corresponding to radiolabeled acyl-CoA molecular species were collected and the radioactivity quantified for palmitoyl-CoA or arachidonoyl-CoA peaks. The mass of both acyl-CoAs was calculated from standard curves plotting the peak area vs the mass of acyl-CoA, where the mass was determined by absorbance at 260 nm using a millimolar extinction coefficient of 15.4. Peak areas were determined with a Beckman System Gold Data system. In separate analyses, the mass/g wet tissue of individual molecular species of total acyl-CoA were calculated based on comparison of peak areas of endogenous molecular species of acyl-CoA with those of the internal standards and relating this value to the mass of brain analyzed (Deutsch et al., 1996, 1997).

2. Analysis of unesterified fatty acids: Quantification of radiolabeled unesterified fatty acids in the plasma (10 μL) was performed by Folch's extraction (Folch et al., 1957) followed by the determination of the radioactivity in the different organic, aqueous, and protein fractions. Radiolabel was shown to be associated completely with the unesterified fatty acid fraction by TLC. Unlabeled free fatty acids in the plasma (30 μL) were isolated by Folch extraction following addition of 1.25 μg pentadecanoic (15:0) and tricosanoic acid (23:0) as internal standards. Diazomethane was added to the dried-down organic phase to convert free fatty acids to methyl esters for subsequent analysis by gas chromatography (Pace-Asciak, 1989). Quantification of fatty acids in brain was performed by similar extraction (Folch et al., 1957) with internal standard followed by resolution of the unesterified free fatty acid fraction by TLC. The sample was eluted with chloroform:methanol (1:1), derivatized by diazomethane, and analyzed by gas chromatography (Deutsch et al., 1997).

Calculations

The rate of incorporation (J_{FA}) of fatty acid into membrane lipids is expressed as follows:

$$J_{FA} = c^*_{br}(T) / \lambda \int_0^T (c^*_{pl} / c_{pl})dt \quad (1)$$

where $c^*_{br}(T)$ is the concentration of radioactive fatty acid incorporated into brain phospholipids at time t = T after iv injection at time zero, c_{pl} and c^*_{pl} represent unlabeled and labeled unesterified fatty acid concentrations in plasma respectively, and λ is the ratio of fatty acid specific activity in the brain acyl-CoA pool to specific activity of the same fatty acid in the plasma unesterified fatty acid pool at a steady-state plasma tracer concentration. The concentration of AA is elevated 30-fold and that of arachidonoyl-CoA 5-6 times in the gerbil 5-min ischemia-reperfusion model (Rabin et al., 1997). Pam and palmitoyl-CoA are elevated to a lesser degree. Dilution of a radiolabeled fatty acid entering the brain acyl-CoA pool from plasma by unlabeled fatty acid recycled in brain can be ascertained and used to calculate the rate of incorporation (J_{FA}; using Eq. 1) of the unlabeled fatty acid from the acyl-CoA precursor pool into phospholipid (Robinson et al., 1992).

Fatty acid incorporation is independent of cerebral blood flow (Robinson et al., 1992; Yamazaki et al., 1994). Thus, regional incorporation of radiolabeled fatty acid reflects local phospholipid metabolism. To quantify regional incorporation, normalized regional incorporation coefficients were calculated as percent from the following expression:

$$k^*_{norm,br} = \left(\frac{c^*_{br}}{Avg[c^*_{ref}]} \right) \times (100) \quad (2)$$

where c^*_{br} is brain radioactivity incorporated in phospholipid in the region of interest, and the denominator is mean brain radioactivity for the nine cerebellar reference regions not affected by ischemia, shown in Table 1. This approach has been used previously (Tone et al., 1987).

Brain concentrations of unesterified fatty acids and acyl-CoA reach a maximum during ischemia, and fall slowly to control levels within 1–2 h (Rehncrona et al., 1982; Nakano et al., 1990; Deutsch et al., 1997; Rabin et al., 1997, 1998; unpublished observations). The diffusion of unesterified radiolabeled fatty acid from plasma into brain across the blood–brain barrier in either postperfusion or control animals is independent of cerebral blood flow, and is determined by the rate of dissociation of fatty acid from plasma albumin and the rate of appearance of lysolipid (Robinson et al., 1992; Yamazaki et al., 1994). Furthermore, for both [^{3}H]Pam and [^{3}H]AA, a steady state is established within only a

few minutes between plasma radioactivity and radioactivity in both the brain free fatty acid and acyl-CoA pools (Washizaki et al., 1994; Grange et al., 1995). Since this time is much shorter than the time for recovery of normal unlabeled brain concentrations during reperfusion (Rehncrona et al., 1982; Nakano et al., 1990), it is very likely that tracer kinetics following ischemia reach a steady state for calculating values for λ, the steady-state ratio of brain precursor pool specific activity to plasma specific activity.

When specific activities in both pools are at steady state, this ratio equals (Robinson et al., 1992):

$$\lambda = \left[\left(c^*_{br,acyl-CoA}\right)/\left(c_{br,acyl-CoA}\right)\right]/\left[\left(c^*_{pl}\right)/\left(c_{pl}\right)\right] \quad (3)$$

The values $(c^*_{pl})/(c_{pl})$ and $(c^*_{br,acyl\ -\ CoA})/(c_{br,acyl\ -\ CoA})$ were calculated and represent specific activities of plasma unesterified fatty acid and brain acyl-CoA, respectively. c_{pl} is the plasma concentration of unlabeled unesterified fatty acid in nmol/g and c^*_{pl} is the plasma concentration of tracer in dpm/g; $c_{br,acyl\ -\ CoA}$ is the brain concentration of [^{3}H]acyl-CoA in dpm/g, whereas $c_{br,acyl\ -\ CoA}$ is the concentration of brain acyl-CoA in nmol/g.

Unlabeled normalized fatty acid incorporation into brain phospholipids from the brain acyl-CoA pool (J_{FA}) is defined as:

$$J_{FA,norm} = \left[\left(k^*_{norm,br}\right)\left(c_{pl}\right)\right]/\lambda \qquad (\text{nmol/mL}) \quad (4)$$

where $k^*_{norm,br}$ is defined by Eq. 2. Equation 4 is another form of Eq. 1 (Robinson et al., 1992).

Thus, we can define an incorporation ratio $J_{FA,isch}/J_{FA,cont}$ relating unlabeled fatty acid incorporation into brain phospholipid during ischemia/reperfusion to unlabeled incorporation in control animals:

$$J_{FA,isch}\ /\ J_{FA,cont} = \left[\left(k^*_{norm,br}\right)_{isch}\left(\lambda_{cont}\right)\right]/\left[\left(k^*_{norm,br}\right)_{cont}\left(\lambda_{isch}\right)\right] \quad (5)$$

Data for untreated animals were presented previously (Rabin et al., 1997, 1998).

Statistics

Statistical analyses were conducted on means ± SEM, and significant differences ($p < 0.05$) were determined by one-way ANOVA. Student's *t*-test, Welch's test, followed by a Student Newmann Keuls or Bonferroni multiple-comparison tests (Program InStat 2.03, Graphpad Software, San Diego, CA).

RESULTS

Effect of Ginkgo biloba *Extract* (EGb 761) *on Postischemic Regional Incorporation of Fatty Acids*

Autoradiography and Normalized Incorporation Coefficients for Pam and AA

[^{3}H]Pam autoradiographs of coronal sections through the dorsal hippocampus and the cerebellum, obtained from control and ischemic animals treated or not treated with 50 and 150 mg/kg/d of *EGb 761*, are presented in Fig. 1. Differential incorporation of [^{3}H]Pam distinguishes brain regions in forebrain and cerebellar sections in control rats (Noronha et al., 1990). Incorporation of [^{3}H]Pam is clearly decreased in all forebrain regions in the first 5 min of postischemic reperfusion. Treatment with 50 or 150 mg/kg/d of *EGb 761* produced a partial or total recovery of [^{3}H]Pam incorporation into the forebrain. In the cerebellum, where the reference regions were selected, [^{3}H]Pam incorporation remained unaffected by ischemia-reperfusion.

Regional differences in normalized incorporation coefficients ($k^*_{norm,br}$) for both [^{3}H]Pam and [^{3}H]AA were calculated from autoradiographs using Eq. 2, and are presented in Table 1. In control animals, normalized coefficients for incorporation of [^{3}H]Pam into forebrain membranes ranged from 61% in cerebral white matter to 128% in the somatosensory cortex. Values for [^{3}H]AA ranged from 59% in the spinal trigeminal tract to 121% in the ventral cochlear nucleus. A significant effect of 5 min ischemia-reperfusion was observed in the group not treated with *EGb 761* (0 mg/kg/d). Ischemia-reperfusion induced a very significant decrease in the normalized incorporation coefficient of each radiotracer ($p < 0.01$), with an average decrease of 31% for [^{3}H]Pam and of 45% for [^{3}H]AA compared with controls.

Treatment with two doses of *EGb 761*, 50 and 150 mg/kg/d, demonstrated a specific and dose-dependent correction of [^{3}H]Pam incorporation in the ischemic-reperfused forebrain regions. With 50 mg/kg/d, [^{3}H]Pam incorporation was improved in all regions, particularly in hippocampus and thalamic/hypothalamic nuclei. With the 150 mg/kg/d dose, no significant difference was found between ischemia-reperfusion and control groups. In contrast, there was no effect of the *EGb 761* treatment on normalized [^{3}H]AA incorporation, even at the highest dose.

Given the dose-dependent effect of *EGb 761*, further biochemical studies focused on effects of treatment with 150 mg/kg/d on the rate of reincorporation of unesterified fatty acids into brain lysolipids generated during ischemia-reperfusion.

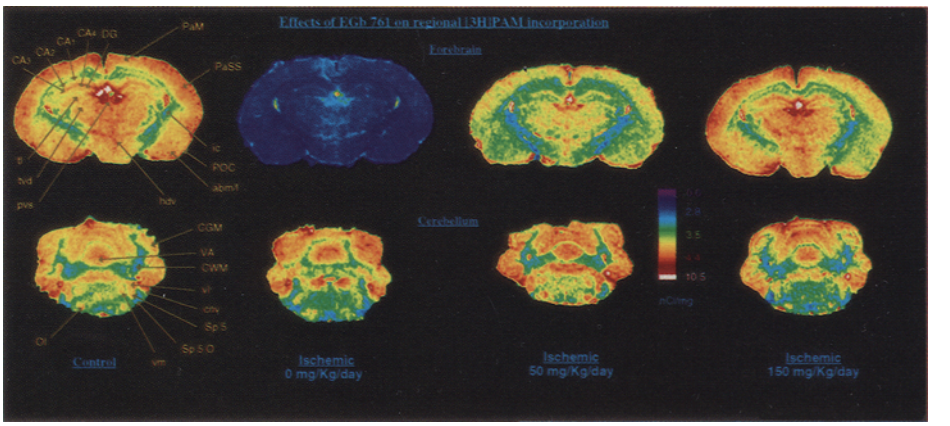

Fig. 1. Digitized color autoradiographs showing regional incorporation of [^{3}H]Pam in forebrain and cerebellar coronal sections of control gerbils, and of ischemic-reperfused animals treated with 0, 50, and 150 mg/kg/d of *Ginkgo biloba* extract (*EGb 761*). Regions in forebrain sections and in the cerebellum (between 1.5 and 1.7 mm, and 5.3 mm posterior to bregma, respectively) were identified from gerbil and rat stereotaxic atlas (Loskota et al., 1974; Paxinos and Watson, 1986). PaSS somatosensory cortex; Pam, frontoparietal motor cortex; PO, primary olfactory cortex; CA1, CA2, CA3, CA4, subfields of the dorsal hippocampus; DG, dentate gyrus; ic, internal capsule; pvs, thalamic paraventricular nucleus; tvd, ventroposterior thalamic nucleus; tl, postthalamic nucleus; abm/l, basolateral and basomedian amygdaloid nuclei; hdv, dorsolateral hypothalamic nucleus; VA, vermis anterior; vl, lateral vestibular nucleus; cnv, ventral cochlear nucleus; vm, median vestibular nucleus; Sp 5, spinal trigeminal tract; Sp 5 O, spinal trigeminal nucleus oral; OI, olive inferior; CGM, cerebellar grey matter; CWM, cerebellar white matter.

Dilution (λ) of Radiolabeled Fatty Acid in Brain Precursor Pools

Concentration of Brain Precursor Pools

In Table 2, concentrations of the different molecular species of brain unesterified fatty acids and acyl-CoAs are shown for both control animals and animals following ischemia-reperfusion, treated or not with 150 mg/kg *EGb 761*. Unesterified fatty acids were considerably increased in ischemia-reperfusion brains, with the relative increase in arachidonic acid being the highest. There was no significant difference in treated vs nontreated animals. Acyl-CoA showed a varied response in reperfusion-ischemia gerbils with both palmitoyl-CoA and docosahexaenoyl-CoA being significantly reduced. However, stearoyl-CoA and arachidonoyl-CoA were significantly increased, with the relative increase in arachi-

Table 2
Brain Free Fatty Acid and Acyl-CoA Concentrations After 5 min Ischemia-Reperfusion and After Treatment with EGb 761 150 mg/kg[a]

Concentration (nmol/g wet tissue)	Control (17)[b]	Ischemia-reperfusion (8)	Ischemia-reperfusion EGb 761 treated (7)
Unesterified fatty acid			
16:0	34.30 ± 2.12	81.16 ± 10.03**	96.78 ± 11.04
16:1	1.86 ± 0.36	1.24 ± 0.41	2.09 ± 0.29
18:0	35.93 ± 4.08	199.01 ± 25.96***	206.45 ± 26.56
18:1	15.02 ± 2.22	49.25 ± 8.62**	45.36 ± 9.52
18:2	3.83 ± 0.79	8.62 ± 1.45*	7.13 ± 1.93
18:3	1.08 ± 0.36	1.68 ± 0.39	1.47 ± 0.55
20:4	3.38 ± 1.21	94.76 ± 20.37**	115.38 ± 28.13
22:6	1.19 ± 0.44	16.26 ± 4.00**	12.69 ± 5.72

Concentration (nmol/g wet tissue)	Control (13)	Ischemia-reperfusion (7)	Ischemia-reperfusion EGb 761 treated (6)
Acyl-CoA molecular species			
16:0	11.72 ± 0.76	8.76 ± 1.02*	8.29 ± 1.61
18:0	3.45 ± 0.27	5.03 ± 0.48*	4.21 ± 0.98
18:1	10.80 ± 0.91	9.98 ± 1.12	8.07 ± 1.11
18.2	1.34 ± 0.20	0.55 ± 0.17**	0.76 ± 0.43
20:4	1.39 ± 0.19	7.60 ± 0.69***	5.49 ± 1.27
22:6	1.30 ± 0.16	0.32 ± 0.05***	0.31 ± 0.10

[a]Each Value is Mean ± SEM. Statistical analysis by Welch's test (Programme Instat 2.03): Significant differences of control versus ischemia-reperfusion values indicated by $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. There was no significant difference between control values of the treated compared to nontreated group so values were combined. There was no significant difference in values for treated compared to nontreated ischemia-reperfusion animals.
[b]Number of animals.

donoyl-CoA being the highest. Again, there was no significant difference between treated and nontreated animals.

Plasma Specific Activity

Constant plasma specific activity of tracer was achieved in the vascular compartment within 30 s, using a computer-controlled pump. More than 92% of the tracer delivered over the 5-min infusion remained unmetabolized (Washizaki et al., 1994), and neither ischemia-reperfusion nor treatment with *EGb 761* modified the distribution of the tracer in the organic, aqueous, or protein fraction of plasma (data not shown). In plasma, radioactivity averaged 41.0 ± 3.2 × 10^6 (n = 30) dpm/mL for [^{3}H]Pam and 30.2 ± 0.2 × 10^6 (n = 27) dpm/mL for [^{3}H]AA.

Plasma concentrations of the different unesterified fatty acid molecular species (Table 3) were determined from the average of arterial

Table 3
Plasma Free Fatty Acid Concentrations With or Without Ischemia and EGb 761 (150 mg/kg/d) Treatment[a]

	Control (11)[b]	Ischemia-reperfusion (16)	Control EGb 761 treated (15)	Ischemia-reperfusion EGb 761 treated (14)
Unesterified fatty acid		nmol/ml plasma		
Palmitic acid (16:0)	164.0 ± 10.8	138.2 ± 10.7	160.1 ± 9.8	148.6 ± 12.8
Palmitoleic acid (16:1)	32.7 ± 4.3	23.1 ± 3.4	24.8 ± 2.6	22.6 ± 2.7
Stearic acid (18:0)	53.9 ± 2.6	48.6 ± 2.2	54.4 ± 2.4	52.6 ± 2.5
Oleic acid (18:1)	174.2 ± 14.7	141.9 ± 14.8	146.5 ± 11.8	140.1 ± 12.4
Linoleic acid (18:2)	230.8 ± 22.4	183.9 ± 12.9*	192.1 ± 14.0	185.7 ± 15.8
Linolenic acid (18:3)	28.0 ± 3.0	23.0 ± 2.0	22.6 ± 1.9	22.5 ± 1.9
Arachidonic acid (20:4)	9.0 ± 0.5	8.0 ± 0.2*	8.7 ± 0.5	7.7 ± 0.4
Docosahexaenoic acid (22:6)	24.7 ± 1.3	22.0 ± 1.6	22.9 ± 1.5	21.2 ± 0.8
Total free fatty acids	717.3 ± 48.3	588.6 ± 44.4	632.0 ± 41.0	601.2 ± 48.0

[a]Each value is mean ± SEM. Statistical analysis by ANOVA. Significant differences between nontreated control and nontreated ischemia-reperfusion groups indicated by $*p < 0.05$, otherwise ns. There was no significant difference between EGb 761 treated control and nontreated control, and between EGb 761 treated control and EGb-761 treated ischemic-reprefusion values for any molecular species.

[b]Number of animals in the group.

blood aliquots sampled just before the start and end of tracer infusion. There was no significant difference in these two values, indicating no effect of tracer infusion on unesterified free fatty acid levels. A small but significant decrease was seen in the linoleic acid and AA levels in the untreated ischemia-reperfusion animals compared with control. No significant difference was found in the total mass of plasma unesterified fatty acids between controls and ischemia-reperfusion treated or nontreated groups, and no other difference in unesterified fatty acid concentrations was noted.

Radioactivity measured in plasma during experiments confirmed that the rate of [^{3}H]Pam and [^{3}H]AA infusion established steady-state levels of tracer in the vascular compartment, even in ischemia-reperfusion animals (treated or not). Average plasma specific activity for [^{3}H]Pam ($n = 8$) and [^{3}H]AA ($n = 7$) was $37.2 \pm 7.3 \times 10^4$ dpm/nmol

and 393.8 ± 46.8 × 10^4 dpm/nmol, respectively. Nontreated controls had similar values. Following ischemia-reperfusion, average plasma specific activity for [^{3}H]Pam was 26.5 ± 6.4 × 10^4 dpm/nmol (n = 9) for nontreated animals and 19.5 ± 2.2 × 10^4 dpm/nmol (n=7) for *EGb 761*-treated animals. Postischemic [^{3}H]AA specific activity was 365.9 ± 42.8 × 10^4 dpm/nmol (n = 7) for nontreated animals and 399.7 ± 58.6 × 10^4 dpm/nmol (n = 8) for animals treated with 150 mg/kg/day of *EGb 761*.

Brain acyl-CoA Specific Activity

Calculation of λ, the dilution factor in the brain acyl-CoA pool for [^{3}H]Pam or [^{3}H]AA (Eq. 3), requires determination of tracer specific activity in the brain acyl-CoA pool and in the plasma unesterified fatty acid pool for each animal. Determination of radioactivity in acyl-CoA peaks isolated by HPLC gave specific activity values for controls of 8.6 ± 1.2 × 10^3 dpm/nmol (n = 8) for palmitoyl-CoA and 42.1 ± 11.7 × 10^3 dpm/nmol (n = 7) for arachidonoyl-CoA. Similarly, in the 5-min ischemia-reperfusion animals, values were 3.7 ± 0.3 × 10^3 dpm/nmol for palmitoyl-CoA (n = 9) and 7.4 ± 4.1 x 10^3 dpm/nmol for arachidonoyl-CoA (n = 4). In the ischemic-reperfused group treated with 150 mg/kg/d of *EGb 761*, palmitoyl-CoA specific activity was 4.5 ± 0.5 × 10^3 dpm/nmol (n = 6) and arachidonoyl-CoA specific activity was 4.9 ± 0.6 × 10^3 dpm/nmol (n = 8), respectively.

Dilution Factor (λ)

λ for [^{3}H]Pam and [^{3}H]AA was determined using Eq. 3 with parameters determined above. Results are summarized in Table 4. During 5 min of reperfusion following the ischemic event, λ was significantly reduced for both radiolabeled fatty acids compared with control animals in the absence of prior treatment with *EGb 761*. λ declined from 0.026 (control) to 0.014 (ischemia-reperfusion) for Pam and from 0.0101 (control) to 0.0017 (ischemia-reperfusion) for AA. There was no significant difference in control values for either fatty acid radiolabel in treated vs nontreated groups. In ischemia-reperfusion gerbils, treatment with 150 mg/kg/d of *EGb 761* restored λ to the control level specifically for Pam, whereas λ for AA stayed at <20% of the treated control with a value of 0.0011.

Normalized Rate of Incorporation of Unesterified Fatty Acids With or Without EGb 761 *Treatment*

Relative rates of incorporation of unesterified fatty acids from the acyl-CoA compartment into brain membranes can be calculated from Eq. 4,

Table 4
Dilution Factor (λ) With or Without Ischemia-Reperfusion and EGb 761 Treatment (150 mg/kg/d)[a]

	Control (6)[b]	Ischemia-reperfusion (9)	Control EGb 761 treated (7)	Ischemia-reperfusion EGb 761 treated (6)
λ Palmitate	0.026 ± 0.004	0.014 ± 0.003** (–50%)	0.028 ± 0.003	0.022 ± 0.006 (–21%)
	Control (6)	Ischemia-reperfusion (4)	Control EGb 761 treated (7)	Ischemia-reperfusion EGb 761 treated (8)
λ Arachidonate	0.0101 ± 0.0014	0.0017 ± 0.0009** (–83%)	0.0095 ± 0.0012	0.0011 ± 0.0008** (–88%)

[a]Each value is mean ± SEM. Statistical analysis by ANOVA followed by Dunnett's test. Significant differences indicated for comparison between control group and ischemia-reperfusion groups by $^{**}p < 0.01$ of either treated or nontreated animals. There was no significant difference between the controls or the ischemic-reprefusion values of the treated compared with nontreated animals.

[b]Number of animals in the group.

$J_{FA,norm}$, the normalized regional incorporation of tracer corrected for dilution of tracer in the precursor pool (λ). Results are presented in Tables 5 and 6. Although *EGb 761* had a dose-dependent effect on the normalized [^{3}H]Pam incorporation coefficient ($k^*_{norm,br}$; Table 1) in ischemia-reperfusion animals, it did not influence the normalized rate of incorporation of unlabeled Pam from palmitoyl-CoA ($J_{FA,norm}$) (Table 5). The incorporation ratio ($J_{FAisch,TR}/J_{FAcont,TR}$; Eq. 5) remained close to 1, either with 50 or 150 mg/kg/d of *EGb 761*.

In control gerbils, normalized unlabeled AA incorporation from AA-CoA ($J_{FA,norm}$) ranged from 6.2×10^4 in the internal capsule to 11×10^4 in the somatosensory cortex, compared with 45.1×10^4 and 75×10^4 in the same regions for Pam. In contrast to Pam, the normalized incorporation of unlabeled arachidonate was augmented in ischemia-reperfused animals compared with control. The ischemia-reperfusion incorporation ratio (J_{FAisch}/J_{FAcont}; Eq. 5) for AA was almost threefold higher than the control ratio. Furthermore, in the ischemic-reperfused group treated by 150 mg/kg/d of *EGb 761*, the ratio was further significantly increased to an average of 4.2-fold over the ischemic nontreated group, and was significantly elevated compared with the value in the nontreated ischemia-reperfusion group.

Table 5
Normalized Incorporation $J_{FA,norm}$ of Palmitate from the Palmitoyl-CoA Pool
Before and After Ischemia-Reperfusion and With or Without EGb 761 Treatment (150 mg/kg/d)[a]

	Palmitate				
Brain regions	$J_{FA,norm,cont}$ (10)[b]	$J_{FA,norm,isch}$ (8)	$J_{FA,norm,isch,TR}$ (5)	JFA_{isch}/JFA_{cont}	$JFA_{isch,TR}/JFA_{cont,TR}$
Anterior circulation			Units × 10^4		
Frontoparietal cortex motor	70.9 ± 9.0	75.0 ± 9.8	73.6 ± 10.9	1.06	1.04
Somatosensory cortex	75.0 ± 9.7	77.0 ± 9.4	83.1 ± 13.3	1.03	1.11
Primary olfactory cortex	59.2 ± 7.6	70.1 ± 9.4	62.1 ± 10.5	1.18	1.05
Hippocampus					
CA1	56.2 ± 7.1	66.1 ± 9.1	59.4 ± 9.8	1.18	1.06
CA2	55.1 ± 7.0	67.1 ± 9.2	56.1 ± 8.4	1.22	1.02
CA3	56.8 ± 7.2	67.1 ± 8.6	58.8 ± 8.7	1.18	1.04
CA4	60.9 ± 7.7	71.1 ± 8.9	62.1 ± 9.2	1.17	1.02
Dentate gyrus	64.4 ± 8.1	76.0 ± 9.4	68.2 ± 10.2	1.18	1.06
Internal capsule	45.1 ± 5.9	47.4 ± 7.7	43.2 ± 6.3	1.05	0.96
Thalam. paravent. nucl.	67.9 ± 8.7	87.9 ± 10.9	75.7 ± 11.2	1.29	1.11
Ventropost. thalam. nucl.	66.2 ± 8.6	75.0 ± 9.3	70.2 ± 10.7	1.13	1.06
Post-thalam. nucl. group	66.8 ± 8.7	79.0 ± 9.1	69.6 ± 11.4	1.18	1.04
Dorsolateral hypothalam. nucl.	60.3 ± 7.6	80.9 ± 9.8	70.2 ± 9.8	1.34	1.16
Basolat-basomed. amygdala	58.6 ± 7.4	70.1 ± 9.4	60.8 ± 9.6	1.20	1.04

[a]Each value is mean ± SEM; TR, treatment with 150 mg/kg EGb 761. Statistical analysis between groups all nonsignificant by ANOVA (Program InStat 2.03). $J_{FA,norm,cont}$, $J_{FA,norm,isch}$, and $J_{FA,norm,isch,TR}$ are defined by Eq. 4 while JFA_{isch}/JFA_{cont} and $JFA_{isch,TR}/JFA_{cont,TR}$ are defined by Eq. 5.
[b]Number of animals in each group.

Table 6
Normalized Incorporation $J_{FA,norm}$ of Arachidonate from the Arachidonoyl-CoA Pool Before and After Ischemia-Reperfusion and With or Without EGb 761 Treatment (150 mg/kg/d)[a]

Brain regions	Arachidonate				
	$J_{FA,norm,cont}$ (10)[b]	$J_{FA,norm,isch}$ (4)	$J_{FA,norm,isch,TR}$ (4)	JFA_{isch}/JFA_{cont}	$JFA_{isch,TR}/JFA_{cont,TR}$*
Anterior circulation			Units × 10^4		
Frontoparietal cortex motor	10.5 ± 1.4	25.4 ± 3.9**	37.8 ± 3.4***,°	2.42	3.60
Somatosensory cortex	11.0 ± 1.5	27.8 ± 4.4**	42.0 ± 3.7***,°°	2.53	3.82
Primary olfactory cortex	8.62 ± 1.2	19.8 ± 3.5**	34.3 ± 2.8***,°°	2.30	3.98
Hippocampus					
CA1	8.91 ± 1.2	23.5 ± 4.1**	32.9 ± 3.2***,°	2.64	3.69
CA2	9.00 ± 1.2	23.1 ± 3.7**	34.3 ± 2.8***,°	2.57	3.81
CA3	8.91 ± 1.2	23.5 ± 3.7***	36.4 ± 3.0***,°°	2.64	4.09
CA4	9.38 ± 1.3	24.5 ± 4.2**	37.1 ± 3.0***,°°	2.61	3.96
Dentate gyrus	9.76 ± 1.3	27.8 ± 4.0***	39.9 ± 3.2***,°	2.84	4.09
Internal capsule	6.16 ± 0.9	16.9 ± 3.7*	28.7 ± 4.0***,°	2.74	4.66
Thalam. paravent. nucl.	10.9 ± 1.5	39.1 ± 5.3***	54.6 ± 4.5***,°	3.59	5.01
Ventropost. thalam. nucl.	11.0 ± 1.5	32.0 ± 4.7***	46.9 ± 3.4***,°°	2.91	4.26
Post-thalam. nucl. group	10.7 ± 1.4	31.6 ± 4.7***	44.1 ± 3.5***,°	2.95	4.12
Dorsolateral hypothalam. nucl.	10.0 ± 1.4	32.5 ± 4.4***	49.0 ± 3.8***,°°	3.25	4.90
Basolat-basomed. amygdala	9.00 ± 1.2	22.1 ± 3.6**	37.8 ± 3.1***,°°	2.46	4.20

[a]Each value is mean ± SEM; TR, treatment with 150 mg/kg EGb 761.Statistical analysis by ANOVA followed by Student-Newman-Keuls multiple comparisons test (Program InStat 2.03). Comparisons between control group and ischemic groups *$p < 0.01$, **$p < 0.01$, and ***$p < 0.001$. Comparisons between ischemic groups °$p < 0.05$ and °°$p < 0.01$. $J_{FA,norm,cont}$, $J_{FA,norm,isch}$, and $J_{FA,norm,isch,TR}$ are defined by Eq. 4 while JFA_{isch}/JFA_{cont} and $JFA_{isch,TR}/JFA_{cont,TR}$ are defined by Eq. 5.
[b]Number of animals in each group.

DISCUSSION

Brain ischemia and the early phase of brain reperfusion following transient ischemia are characterized by phospholipase activation and massive release of unesterified fatty acids, especially AA, and of platelet-activating factor (Doucet and Bazan, 1993; Bazan et al., 1993). Some drugs have been shown to reduce this release or attenuate its pathological consequences on cell metabolism. For example, investigators have reported decreased accumulation of AA in ischemic models following administration of an antagonist to platelet-activating factor (Birkle et al., 1988; Bazan et al., 1993). Other investigators have reported that peripheral administration of mepacrine, a phospholipase A_2 inhibitor, reduces the release of excitotoxic amino acids during cerebral ischemia (O'Regan et al., 1995). In addition, cyclooxygenase and lipoxygenase metabolites, namely prostaglandins and leukotrienes, are elevated in ischemia and have been linked to modulation of neurotransmission, vascular permeability, and cerebral blood flow (Pickard, 1981; Moskowitz et al., 1984). Inhibition of cyclooxygenase and lipoxygenase enzymes has been linked to a decrease in delayed neuronal death in the gerbil hippocampus (Nakagomi et al., 1989). Diversion of AA from these enzymatic pathways to reacylation of lysolipid should ameliorate these effects and stabilize the membrane bilayer. Furthermore, platelet-activating factor and perhaps polyunsaturated fatty acids are involved in activation of immediate early genes (Bazan et al., 1991; Doucet and Bazan, 1993; Takemodo et al., 1995) and delayed neuronal death. Therefore, attenuation of AA and platelet-activating factor concentrations can modulate activation of these genes.

In this study, we used quantitative autoradiography and biochemical analysis to determine regional incorporation of unlabeled fatty acid from the brain acyl-CoA precursor pools into membrane phospholipids, as well as dilution of specific activities of these pools following ischemia. Combination of autoradiographic techniques with peripheral infusion of radiolabeled fatty acids has been used to demonstrate regional alterations and remodeling of phospholipid metabolism in the brain (Robinson et al., 1992). We monitored the effect of *EGb 761* to try to understand its reported neuroprotective effect during ischemia-reperfusion in awake gerbils.

Normalized incorporation coefficients ($k^*_{norm,br}$) for [^{3}H]Pam and [^{3}H]AA from blood were significantly reduced in all ischemic regions by 31 and 45%, respectively, in the early phase of reperfusion in untreated animals (Table 1). These reductions reflect dilution of vascular-derived tracer by unlabeled unesterified fatty acids released during ischemia (Bazan, 1970; Ikeda et al., 1986; Yoshida et al., 1986; Rabin et al., 1997). Treatment with 50 and 150 mg/kg/d of *EGb 761* orally for 14 d demonstrated a dose-dependent recovery of [^{3}H]Pam incorporation to reach the control level (Fig. 1, Table 1). Postischemic incorporation of [^{3}H]AA was unaffected by *EGb 761*, with incorporation coefficients like those in

untreated animals. In contrast to the effect of *EGb 761* on reincorporation of [^{3}H]Pam in ischemia-reperfusion, the effect of treatment on $J_{FA,norm}$ gave no increase over control levels. Thus, a decrease in the dilution of [^{3}H]Pam radiolabel after drug treatment (Fig. 1, Table 1) may be owing to a direct effect of *EGb 761* on phospholipase A_1. Modulation by *EGb 761* of phospholipase activity, including phospholipases A_1 and A_2, as previously indicated in ischemia and other cerebral pathologies (Bazan and Rodriguez de Turco, 1992; Rodriguez de Turco et al., 1993), cannot be ruled out, but additional experiments failed to demonstrate effects of treatment on concentrations of brain unesterified palmitate or palmitoyl-CoA in postischemic brain.

The dilution factors (λ) for the two radiolabeled tracers in the brain acyl-CoA precursor pool of control rats have been quantified previously (Washizaki et al., 1994; Grange et al., 1995) and agree with values for control animals presented here. Values of λ for [^{3}H]Pam and [^{3}H]AA in ischemic-reperfused gerbils (Table 4) were decreased by 50 and 80%, respectively, following ischemia-reperfusion. Treatment with 150 g/kg/d *EGb 761* for 14 d did not significantly affect λ for [^{3}H]AA, but returned λ for [^{3}H]Pam to the control value. This effect on λ likely accounts for net return of tracer incorporation to control levels. Because of a lower λ owing to the release of Pam during ischemia while J_{FA} was maintained, less tracer from plasma was reincorporated into brain phospholipids. Treatment with *EGb 761* reversed these effects specifically for Pam.

Recovery of normal brain concentrations of unesterified fatty acids and acyl-CoAs occurs within 1 h following the onset of reperfusion after ischemia (Rehncrona et al., 1982; Nakano et al., 1990; unpublished results). To calculate the rate of reincorporation of unesterified fatty acids into membrane phospholipids via acyl-CoA at 5 min postischemia, a normalized incorporation rate ($J_{FA,norm}$) was obtained from the normalized incorporation coefficient of tracer determined by quantitative autoradiography and from λ quantified by biochemical analysis (Eq. 3). The dilution factor λ can be used to determine incorporation of unlabeled fatty acid in all forebrain regions, since all forebrain regions are equally affected by ischemia-reperfusion. Interestingly, $J_{FA,norm}$ of unesterified Pam was not affected in the early phase of reperfusion and remained similar to the value for control animals not affected by pathology (Table 5). Similarly, *EGb 761* treatment did not change $J_{FA,norm}$ of Pam as indicated by a $J_{FA,isch}/J_{FA,cont}$ ratio of approximately 1. However, $J_{FA,norm}$ for AA was markedly augmented by two- to threefold in ischemia-reperfused animals (Table 6). Treatment with the *EGb 761* further elevated incorporation to 4.2-fold on average compared with control, showing a selective effect of treatment. Increased $J_{FA,norm}$ for arachidonate into the membrane bilayer from the arachidonoyl-CoA pool would reduce AA conversion into cyclooxygenase and lipoxygenase metabolites. The effect of *EGb 761* treatment on AA incorporation is schematically presented in Fig 2.

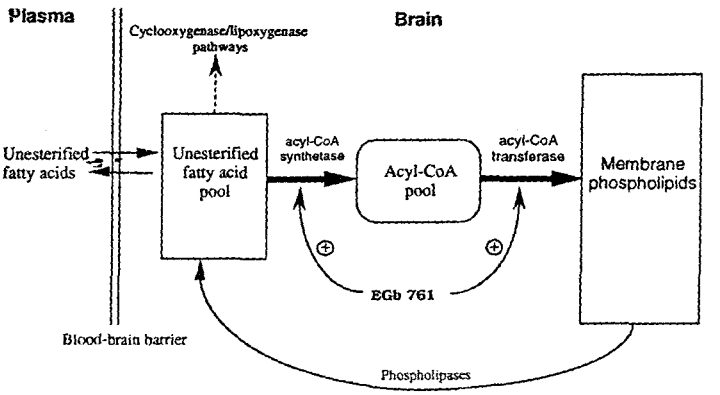

Fig. 2. Schematic representation of unesterified fatty acid uptake from plasma into brain phospholipids. Diagram shows the relation between the different vascular and cerebral pools involved in unesterified fatty acid incorporation as well as the effect of *EGb 761* on the reincorporation process.

Results obtained with quantitative autoradiography support, in part, previous reports linking effects of *EGb 761* to decreased fatty acid release and to modulation of activation of phospholipases (Bazan and Rodriguez de Turco, 1992). At a dose equivalent to the human therapeutic dose (50 mg/kg/d), *EGb 761* reduced release of Pam during ischemia or reperfusion. Thus, phospholipase inhibition may contribute to our results with *EGb 761*, but its significance appears on the reincorporation of unesterified AA into brain phospholipid.

AA has been shown to inhibit glutamate reuptake by glial cells (Barbour et al., 1989; Volterra 1994) and to potentiate the NMDA-evoked current in neurons (Miller et al., 1992). AA is released during activation of a number of receptor subtypes, such as NMDA receptors (Lazarewicz et al., 1988), or during simultaneous activation of metabotropic and AMPA receptor activation by glutamate (Dumuis et al., 1988). AA also can increase the release of glutamate from hippocampal synaptosomes (Lynch and Voss, 1990). Thus, high levels of AA release during ischemia-reperfusion may contribute to the excitotoxic effect of glutamate by extending exposure to this amino acid at the synapse. In turn, persistent unesterified AA during reperfusion could generate reactive oxygen species, which damage brain parenchyma (Chan et al., 1982). Significant protection from ischemia-reperfusion injury by free radical scavenging drugs (Watanabe and Egawa, 1994) and superoxide dismutase (Matsumiya et al., 1991) suggests a role for free radical mechanisms.

Increased reincorporation of AA owing to *EGb 761* treatment may affect upregulation of acyl-CoA synthetase/acyltransferase enzymes responsible for reincorporation of AA into the membrane bilayer. Upregulation would help to decrease AA concentration more rapidly and increase the rate of reacylation of membrane-destabilizing lysolipids. If the protective effect of *EGb 761* is owing to upregulation of the AA rea-

cylation in brain, this mechanism may be a target for further pharmacological intervention during sustained ischemia-reperfusion.

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