Life Sciences, Vol. 58, No. 16, pp. 1315-1321, 1996

0024-3205/96 $15.00 + .00

ELSEVIER

PII S0024-3205(96)00097-5

EXTRACTS OF GINKGO BILOBA LEAVES INHIBIT MONOAMINE OXIDASE

Helen L. White, Philip W. Scates and Barrett R. Cooper

Division of Pharmacology, Burroughs Wellcome Co
Research Triangle Park, NC 27709

(Received in final form February 12, 1996)

Summary

Extracts of Ginkgo biloba leaves produce reversible inhibition of rat brain monoamine oxidase (MAO). Both MAO-A and -B types were inhibited to a similar extent. The MAO inhibitory compound(s) were present in dried or fresh Ginkgo biloba leaves as well as in commercially available capsules of Ginkgo biloba and appear to be heat stable with relatively low molecular weight. MAO inhibition by Ginkgo biloba may be a mechanism underlying reported anti-stress and anxiolytic activities of this natural product.

Key Words: monoamine oxidase, Ginkgo biloba, stress, anxiety

Ginkgo biloba leaves have been used for medicinal purposes for hundreds of years in China. More recently the popularity of this natural plant product has spread throughout Europe, where it is now widely prescribed for problems relating to cerebral and vascular insufficiency, cognitive deficits, and other age-associated impairments. The chemical composition and therapeutic actions of the standardized Ginkgo biloba extract, EGb 761, have been summarized by DeFeudis (1). Dozens of compounds have been isolated from extracts of Ginkgo leaves and identified. These include a variety of alcohols, aldehydes, ketones, acids, terpenes, steroids, catechins, flavonoids, and glycosides (2,3). The possible pharmacological actions of most of these compounds is not yet known. Probably the most well known component of Ginkgo is BN 52021 (ginkgolide B), an antagonist of platelet-activating factor receptors with Ki of 2 μM in human neutrophil preparations (4). This ginkgolide may be responsible for reported effects of Ginkgo on immune responses (5).

More recent studies have explored pharmacological effects of Ginkgo leaf extracts on the central nervous system. Levels of biogenic amines in brains of rats were significantly changed after 7 days of dosing. Serotonin, in particular, was elevated in most brain regions (6). Chronic dosing with Ginkgo extracts has also given positive results in rodent models that are thought to be predictive of anti-anxiety or anti-stress effects in man (7,8). Based on similar animal tests, a patent has been issued on the use of bilobalide, or Ginkgo enriched in this component, for treatment of anxiety or depression in man (9). In the present study, we have explored the effect of Ginkgo leaf extracts on monoamine oxidase (MAO), an enzyme which can regulate brain concentrations of serotonin, norepinephrine, and other biogenic amines. The inhibition of MAO has been shown to produce antidepressant or anxiolytic responses in animal models and in man (12).

Corresponding author: H.L. White, 210 Ridgecrest Drive, Chapel Hill, NC 27514, (919) 968-1051

Methods

MAO assays

MAO-A and -B forms were assayed using the method of White and Glassman (13). Rat brain mitochondrial extract was preincubated with the inhibitor for 15 min at 37°C in 50 mM potassium phosphate buffer (pH 7.4). Substrates [^{3}H]serotonin (0.2 mM, 5 Ci/mol) and [^{14}C]β-phenethylamine (10 μM, 3 Ci/mol) were then added, and incubation at 37°C was continued for 20 min. Blank assays contained 2 mM pargyline to inhibit all MAO activity. The reaction was terminated with 0.2 ml of 2 N HCl, and products were extracted with 6 ml of ethyl acetate/toluene (1:1). A 4 ml aliquot of the organic layer was counted in 10 ml of Ecolite (ICN) in a scintillation spectrometer programmed for double-label counting. Assays were performed in triplicate unless otherwise indicated. At the above concentrations, serotonin is a selective substrate for MAO-A, and β-phenethylamine is a selective substrate for MAO-B. Thus inhibitors of MAO-A or B are detected in the same procedure.

Reversibility of Inhibition

Reversibility of *in vitro* inhibition was determined by dialysis. Spectra/Por membrane tubing (Fisher Scientific) with a 12-14K molecular weight cutoff was used for this purpose. Mixtures of 50 mM potassium phosphate buffer (pH 7.4) and MAO extract +/- aqueous Ginkgo extract were preincubated at 37°C for 15 min. A 1 ml portion of each mixture was then dialyzed at 4°C with shaking *vs* 40 ml of outer buffer (50 mM potassium phosphate, pH 7.4, 5% sucrose, 0.1 mM EDTA, 1 mM dithiothreitol). Outer buffer was replaced with fresh buffer at approximately 3 hr and 19 hr after the start of the dialysis, and dialysis was terminated at 24 hr. Undialyzed portions of each mixture were maintained at 4°C over the same time period. Both dialyzed and undialyzed mixtures were assayed for MAO activity at the same time.

Preparation of Ginkgo Extracts

Dried Ginkgo biloba leaves were mixed with a 10-fold weight ratio of glass distilled water (2 g dried leaves + 20 ml water) and warmed at 50°C for 0.5 hr with constant stirring. The warm mixture was then sonicated for 2 min on setting 9, using a Heat Systems Ultronic Model W385 with cup horn attachment. The 0.5 hr warming was repeated, followed by a second sonication treatment, after which the mixture was centrifuged at 200 g for 10 min to remove large particulate matter. The supernatant was centrifuged at 9000 g for 30 min at 4°C to give a clear golden brown extract. This aqueous extract could be stored at 4°C for at least a week without loss of MAO inhibiting activity. Ethanolic extracts were prepared as above, except that 2 g dried leaves were initially mixed with 10 ml ethanol. Fresh Ginkgo leaves were first chopped and dispersed in glass distilled water using a small mortar and pestle prior to the above warming and sonication steps. Aqueous preparations from Ginkgo capsules were obtained by dispersing each capsule in a 5-fold weight of glass distilled water, warming at 50°C for 0.5 hr with stirring, sonicating for 15 sec, stirring the suspension at room temperature for 2 hr, and then centrifuging at 9000 g for 10 min to produce a light brown supernatant.

Materials

[^{3}H]Serotonin (5-hydroxy [G-^{3}H]tryptamine creatinine sulfate, 9300 Ci/mol) from Amersham and [^{14}C]β-phenethylamine (57 Ci/mol) from New England Nuclear were diluted into deoxygenated (by N_2 bubbling) glass-distilled water with corresponding unlabeled compounds (from Sigma

Chemical Co., St. Louis, MO) to give stock substrate solutions that could be diluted 6-fold into final MAO assay mixtures for *in vitro* studies. All substrate solutions were stored under nitrogen at -20°C. Extraction solvents, toluene and ethyl acetate, were of HPLC grade. Dried Ginkgo leaves were purchased at a local organic foods store, and fresh Ginkgo biloba leaves were obtained from a local nursery. Ginkgo biloba capsules/tablets were from two sources: General Nutrition Corp., Pittsburgh, PA (Natural Brand™) identified as containing 50 mg each of standardized 50:1 leaf extract containing 24% Ginkgo flavone glycosides, the "European standard"; and from Nature's Way, Inc., Springville, Utah (Ginkgold™) containing standardized extract EGb 761. Proteins were determined using a BCA assay kit from Pierce (Rockford, IL). Ginkgolide A (BN52020),which is derived from Ginkgo and is an antagonist of platelet-activating factor (PAF), was purchased from Sigma Chem. Co., St. Louis, MO.

Results

Aqueous extracts of dried Ginkgo biloba leaves inhibited both MAO-A and MAO-B in mitochondrial preparations from rat brain, with no selectivity for either form, as shown in Fig. 1. Because it was not possible to determine the concentration of the inhibitory compound(s) in the extract, the abscissa in Fig. 1 is expressed as the equivalent mg of dried leaves which yielded the

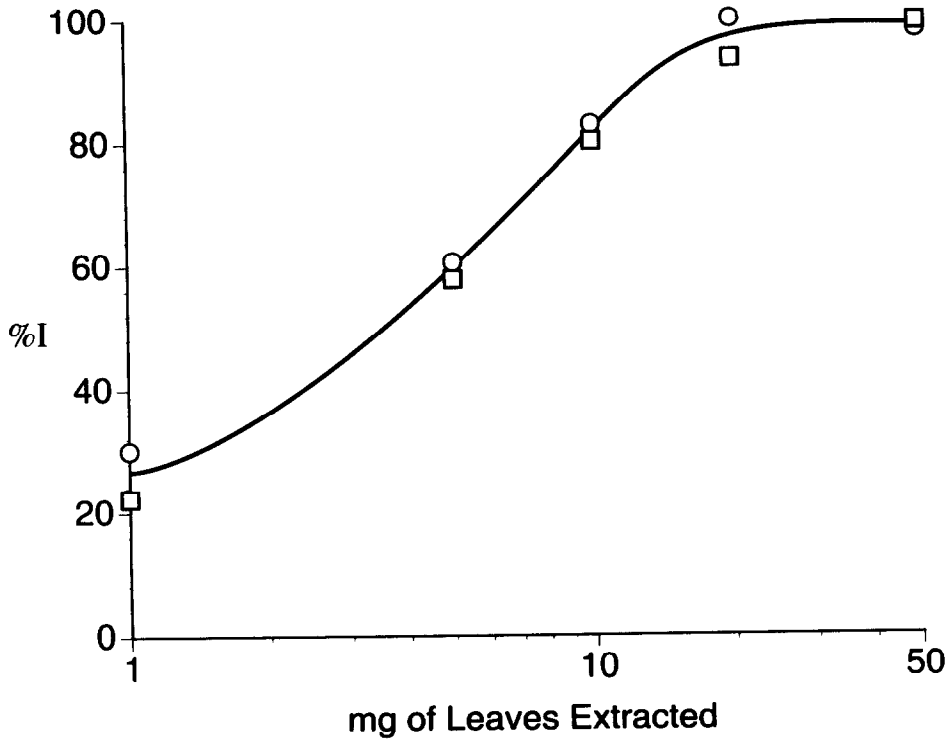

FIG.1

Inhibition of MAO-A (circles) and MAO-B (squares) by an aqueous extract of dried Ginkgo biloba leaves. The concentration of leaf extract is expressed as the weight of leaves which yielded the amount of inhibitory material present in each assay. Each point is the mean of three assays, with SE within the dimension of the symbols.

amount of inhibitor in the assays. Complete inhibition of both MAO-A and MAO-B were achieved when assays contained an amount of extract derived from 20 mg dried leaves.

Following a 15 min preincubation of MAO with aqueous Ginkgo extract at 37°C, the inhibition of both MAO types was reversed by dialysis at 4°C over a 24 hr period, as indicated by data in Table I. The apparent 22% stimulation of MAO-A observed after dialysis suggests a stabilization of the enzyme by the presence of the inhibitor during early stages of the dialysis. Since dialysis membrane having a molecular weight cutoff of 10000 was used in this experiment, the inhibitory component of the Ginkgo extract must have been below 10K, and is not likely to be a protein. This conclusion was further supported by a separate experiment in which the Ginkgo extract was tested for heat stability. Data in Table II shows that the MAO inhibitory component was unaffected by heating at100°C.

TABLE I
Reversibility of MAO Inhibition by Ginkgo Extract

	MAO-A (dpm +/- SE)	% I	MAO-B (dpm +/- SE)	% I
Controls	7585 +/- 695		7574 +/- 102	
Not Dialysed	1267 +/- 56	83.3	984 +/- 17	87.0
Dialysed	9272 +/- 758	-22	6908 +/- 47	8.8

MAO extract was preincubated at 37°C for 15 min +/- aqueous Ginkgo extract (equivalent from 10 mg dried leaves) and then dialyzed for 24 hr at 4°C. (n=3)

TABLE II
Heat Stability of MAO Inhibiting Factor

	Percent Inhibition +/- SE	
	MAO-A	MAO-B
No Pretreatment	85.0 +/- 0.6	80.6 +/- 0.5
10 min at 100°C	87.5 +/- 1.0	80.7 +/- 1.2

100 µl portions of aqueous Ginkgo extract from dried leaves (equivalent of 10 mg leaves) were heated to dryness at 100 °C in a boiling water bath for 10 min or maintained at 0°C (no pretreatment). The dried extracts were resuspended in 100 µl distilled water to restore initial volume before addition of other components of the enzyme assays. (n=3)

The extraction procedure described in Methods gave reproducible inhibitions with different batches of leaves, either dried or fresh. However, extracts from fresh leaves contained about 8-fold less protein than those from dried leaves (3.9 mg/ml and 31 mg/ml, respectively). Commercial Ginkgo preparations also contained MAO inhibitory compound(s). These contained a standard 50:1 leaf extract which is composed of 24% Ginkgo flavone glycosides, the "European standard". In Table III, the MAO inhibitions obtained with various Ginkgo preparations is expressed as mg equivalent of either dried or fresh leaves or, in the case of commercial products, as mg of the above standard 50:1 extract per assay. Ginkgolide A (90% purity) was also tested and found to be inactive.

TABLE III
MAO Inhibition by Various Ginkgo Preparations

		Percent Inhibition	
		MAO-A	MAO-B
	(mg equivalent)		
Dried leaves; aqueous extract	(5)	54.6 +/-2.8	59.1 +/-1.4
" " " "	(10)	84.2 +/-0.7	80.5 +/-0.4
Dried leaves; ethanol extract	(5)	69.1 +/-1.0	71.6 +/-0.7
Fresh leaves; aqueous extract	(1)	17.1 +/-1.9	5.6 +/-1.4
" " " "	(5)	57.5 +/-1.2	34.6 +/-1.1
" " " "	(10)	78.3 +/-0.4	51.1 +/-1.3
Natural Brand™; aqueous extract	(1.5)	39.2 +/-0.6	48.7 +/- 1.2
Ginkgold™; aqueous extract	(3)	95.7 +/-0.2	88.5 +/-0.3

Extracts were prepared as described in Methods and tested using the standard MAO assay. For leaf extracts, mg equivalent represents the mg of leaves which yielded the amount of extract in the MAO assays. Ethanolic extracts were first dried under nitrogen in the assay tubes and resuspended in an appropriate volume of water before addition of other components. For commercial preparations, mg of standard 50:1 extract per assay is given. Each assay was performed at least in triplicate. Data is expressed as % inhibition +/- SE.

Discussion

Although no specificity for MAO-A or B was shown by extracts of dried leaves, the extract from fresh Ginkgo leaves did appear to inhibit MAO-A somewhat more effectively than MAO-B. This suggests a possibility that more than one inhibitor was extracted from fresh leaves, with at least one component having a greater inhibitory effect on MAO-A. On the basis of protein assays of fresh and dried leaf extracts, one might assume that the latter were about 8-fold more concentrated. This is a reasonable conclusion since the fresh leaves included a substantial moisture content. Therefore, it appears that the potency of the fresh leaf extract, based on protein content, may have been greater than that from dried leaves.

MAO inhibiting components that could be extracted from Gingko leaves were soluble in either water or ethanol, as suggested by data in Table III. It is of interest that bilobalide, the sesquiterpene lactone component of Ginkgo claimed to have antidepressant and anti-anxiety effects in a recent patent (9), is also both ethanol and water soluble. A variety of flavonoids might have also been present in our aqueous extracts, and some of these are known to have enzyme inhibiting properties (10). However, Weinges et al. (2) reported that they did not detect flavonoids in alcoholic extracts. In addition, proanthocyanidins or polyphenols from plants, including Ginkgo, have been shown to bind to proteins causing inactivation or precipitation (11). This complex formation was not reversed by dialysis. Since the MAO inhibition by Ginkgo extracts was reversible on dialysis (Table I), it is unlikely that this type of complex formation is involved.

If sufficient inhibition of MAO-A in brain could be obtained by ingestion of Ginkgo teas or capsules, this inhibition would provide a reasonable mechanism for the anti-stress or antidepressant effects observed in animal models (7-9). It would also be consistent with a report (6) that oral

administration of Ginkgo extracts to 26 month old rats for 7 days produced an elevation of serotonin in frontal cortex, hippocampus, striatum, and hypothalamus. Although these authors did not observe increased norepinephrine levels in brain, another study in rats (14) showed that the 0-methylated amine metabolite of norepinephrine, normetanephrine, was markedly elevated in cerebral cortex after 14 days of oral Ginkgo extract administration. This would be expected if MAO inhibition caused a diversion of norepinephrine metabolism toward the catechol O-methyl transferase pathway. These workers also observed a decrease in Bmax values for β-adrenoceptor function by 30 days of treatment, an event that has been associated with the antidepressant effects of moclobemide and other MAO inhibitors (15). A possible involvement of MAO inhibition was also suggested by others, who observed that Ginkgo biloba extracts potentiated norepinephrine-induced contractions in rabbit aortic strips (16).

In a preliminary unpublished study (n=1), we have observed 30 to 50% inhibition of human platelet MAO-B at 1 and 3 hours following the ingestion of 4 Ginkgold capsules. In the same individual, platelet MAO-B was inhibited by 29% at 1 hr after drinking the aqueous extract from 2 g of dried Ginkgo leaves. However, these experiments indicate only that MAO inhibitory components of Ginkgo are available to platelets, and it is not certain that the reversible inhibition observed in the present study will translate into inhibition of MAO in brains of humans at doses of Ginkgo normally used. A recent pharmaco-dynamic study (17) in which Ginkgold was administered to 12 human volunteers indicated that, over an oral dose range of 40 to 240 mg of Ginkgo extract, bioavailability in brain was sufficient to produce an electrophysiological effect similar to that of certain other compounds that are classified as cognitive activators, antidepressants, or anxiolytics. Additional experiments are necessary to determine pharmacokinetic parameters of MAO inhibitory components of Ginkgo, especially during chronic dosing when pharmacological effects can be observed. Although the *in vitro* inhibition observed in the present study appears relatively weak, if brain/plasma ratios become sufficiently high or if active compounds accumulate in tissues during chronic dosing, a substantial inhibition of brain MAO may result.

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