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Protection of Mitochondrial Respiration Activity by Bilobalide

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ABSTRACT. Mitochondria alteration is an early event in ischemia-induced damage, and its prevention improves tissue survival upon reperfusion. Adenine translocase and complex I activities are rapidly affected by ischemia. *Ginkgo biloba* extract demonstrates anti-ischemic properties attributable to the terpenoid fraction, mainly due to the presence of bilobalide. The mechanism of the protection afforded by bilobalide is not yet known. In this work, the effects of bilobalide on mitochondrial respiration were investigated. Mitochondria isolated from rats treated with bilobalide (2 to 8 mg/kg) showed a dose-dependent increase in the respiratory control ratio, due to a lower oxygen consumption during state 4. Bilobalide also decreased the sensitivity of oxygen consumption to inhibition of complex I by Amytal or to inhibition of complex III by antimycin A or myxothiazol. There was no protection of complexes IV and V. It also increased the activity of complex I but not of adenine translocase. Similar effects were also obtained *in vitro* when control mitochondria were preincubated for 1 hr with 0.8 μg/mL bilobalide. Treatment of the rats with 8 mg/kg bilobalide also prevented the ischemia-induced decrease in state 3 of the mitochondrial respiration and thus the decrease in RCR. The protective effect of bilobalide on cellular ATP content observed under ischemic conditions can be correlated with the above observations. By protecting complex I and III activities, bilobalide allows mitochondria to maintain their respiratory activity under ischemic conditions as long as some oxygen is present, thus delaying the onset of ischemia-induced damage. This mechanism provides a possible explanation for the anti-ischemic properties of bilobalide and of *Ginkgo biloba* extract in therapeutic interventions. BIOCHEM PHARMACOL **58**;1: 109–119, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. mitochondrial respiration; state 3; state 4; complex I; complex III; adenine translocase; bilobalide; *Ginkgo biloba* extract

Mitochondria are the main source of energy which sustains cellular metabolism and integrity. The decrease in oxygen supply during ischemia impairs energy production by mitochondria. Cellular and tissue functions can be fully recovered upon reperfusion if the duration of ischemia is relatively short [1]. However, longer periods of ischemia cause irreversible tissue injury. While reperfusion is necessary for the viability of the organ, ischemia-induced damage is exacerbated by reoxygenation during reperfusion. This phenomenon is known as the oxygen paradox [2]. The transition from reversible to irreversible ischemia is partly dependent on the functional state of mitochondria [1, 3], and restoration of oxidative metabolism determines functional recovery [4].

Many mitochondrial alterations have been described in ischemic organs [for reviews see refs. 5 and 6]. Mitochondria extracted from ischemic hearts showed reduced respiratory function due to damage affecting complex I and to a lesser extent complex III [7]. Similar alterations were observed with mitochondria isolated from ischemic brain [8]. F_1F_0 ATPase [9] and the adenine nucleotide translocase [10] have also been reported to be inhibited [11, 12]. Morphological damage such as swelling and loss of the cristae structure was revealed in transmission electron microscopy [13].

There is evidence from experimental and clinical work that EGb 761§ protects brain tissue [14–17] as well as myocardium [18–20] against hypoxic or ischemia/reperfusion damage. It was first proposed that such protection was associated with the free radical scavenging properties of EGb demonstrated in *in vitro* experiments and which could be due to its flavonoid constituents [21, 22]. However, the concentrations of flavonoids needed to achieve such protection are much higher than those obtained during the usual EGb 761 therapeutic dosage. Other(s) active constituent(s) of EGb 761 may thus be responsible for its protective effect. Interestingly, EGb 761 has been shown to protect against the hypoxia-induced decrease in ATP content in cultured endothelial cells. This protective effect

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§ *Abbreviations:* EGb 761, standardized *Ginkgo biloba* extract; mCCP, carbonylcyanide *m*-chlorophenyl hydrazone; RCR, respiratory control ratio; and P/O ratio, ratio of oxidative phosphorylation.

was obtained in the absence of reoxygenation and was due to the main constituent of the non-flavone fraction of EGb, bilobalide, and to a lesser extent to another terpenoid, ginkgolide B [23].

In an attempt to unravel the mechanism of this protection, it was shown that bilobalide did not increase glycolysis activity under hypoxic conditions but rather delayed its activation, probably by preserving ATP regeneration by mitochondria [24]. Indeed, bilobalide was able to increase the respiratory control ratio of mitochondria isolated from liver of rats treated orally [24]. Since hypoxia is known to alter mitochondria respiratory activity, the protection of the cellular ATP content and the delay in glycolysis activation observed in the presence of bilobalide is best explained by a protection of this respiratory activity by bilobalide.

However, the mechanism of action of bilobalide on mitochondrial respiration is not known. In this work, the mechanism whereby bilobalide operates was investigated on mitochondrial respiration in order to understand how it protects mitochondria under ischemic conditions. Its effect on RCR was first studied on mitochondria purified from liver of rats treated orally with various doses for 14 days. The RCR is a direct measure of the coupling of mitochondria and of the efficiency of ATP regeneration from the mitochondrial electron transport chain. It is the ratio between state 3, which is the rate of phosphorylating respiration in the presence of exogenous ADP, and state 4, which is the rate of resting respiration when all ADP has been consumed. The effect of bilobalide was further investigated on the activities of respiratory chain complexes I, III, IV, and V in isolated mitochondria. The results pinpoint a protective effect of bilobalide on complexes I and III.

MATERIALS AND METHODS

Materials

ADP, β-hydroxybutyrate, succinate, glutamate, malate, Amytal, antimycin A, oligomycin, atractyloside, EGTA, myxothiazol, cytochrome *c*, rotenone, and BSA (A7030) were from Sigma Chemical Co. [^{14}C]-ADP (specific activity 55 mCi/mmol) came from Dupont-NEN Products. Dinitrophenol, KCN, $Fe(CN)_3$, and the other chemicals of analytical grade were from Merck. Bilobalide was kindly provided by the Institut Henri Beaufour (Paris, France).

Isolation of Rat Liver Mitochondria

Female Wistar rats (IFFA Credo) were housed in groups of four before the experiments and allowed to acclimatize to their new laboratory conditions for at least 14 days. They were then treated orally for 14 days with bilobalide or distilled water (0.6 mL per day). Rats were fasted for at least 18 hr and after killing, the liver was chilled in a medium containing 0.25 M saccharose, 10 mM EDTA, 10 mM HEPES and 2 g/L BSA. Three g of liver were homogenized by two successive passages in a Teflon homogenizer (Type C, AH Thomas Co.). A nuclear fraction was prepared by a 10-min centrifugation at 754 *g* at 4°. The supernatant was kept at 4°. The pellet was centrifuged again for 10 min at 580 *g* and the supernatant added to the previous one and adjusted to a final volume of 45 mL. Two times eight milliliters were sampled to isolate mitochondria by a 3-min centrifugation at 10,300 *g* (Beckman LS65B ultracentrifuge). Resuspension of the mitochondrial pellet was carried out carefully with a 7-mL Dounce loose (Kontes Glass Co.) in NaCl 7.05 mM, KCl 70.5 mM, K_2HPO_4 5.45 mM, KH_2PO_4 4.55 mM, BSA 0.15%, pH 7.2.

Liver Perfusion

Rat were starved for 18 hr before liver perfusion and were anesthetized by ether for 5 min. The abdomen was opened, the hepatic vein rapidly cannulated, and the perfusion started within 1 min [25]. During perfusion, the liver remained in the abdomen. The perfusion solution was a modified Krebs–Henseleit bicarbonate buffer pH 7.4 [7] kept at 42° in order to obtain 37° in the liver perfusion and continually gassed with either O_2/CO_2 (19:1) for normoxic buffer or N_2/CO_2 (19:1) for anoxic buffer (PO_2 = 10–15 mm Hg). The buffer contained 8 mg/L bilobalide for all livers. All livers were perfused for 5 min at 8 mL/min in circulating mode to equilibrate the tissue, before one of the following conditions was imposed. Controls which had been perfused with normoxic buffer were then continuously perfused with normoxic buffer at 4 mL/min for 10 min. Ischemia was induced by stopping the circulation of the perfusion buffer (anoxic buffer) for 10 min.

Respiration Determination

The rate of oxygen consumption by the mitochondrial fraction was assayed by an oxypolarographic method using a Clark-type electrode. The RCR was calculated according to Chance and Williams [26]: it is the ratio between the oxygen consumption rate in the presence of 5 mM exogenous succinate or 10 mM glutamate/malate and 0.16 mM ADP (state 3) and the rate before or after ADP consumption. States 2 and 4 were similar under our experimental conditions. The P/O ratio was calculated as: $0.0079 \times 2 \times$ O_2 consumption expressed in % of total O_2 consumption obtained by adding yeast to the oxypolarograph chamber.

Mitochondrial Preincubation

Mitochondria prepared from liver were suspended in NaCl 7.05 mM, KCl 70.5 mM, K_2HPO_4 5.45 mM, KH_2PO_4 4.55 mM, BSA 0.15%, pH 7.2 at a final mitochondrial protein concentration of approximately 40 mg/mL. Mitochondria were then incubated for 1 hr at 4° in the absence (control) or the presence of different concentrations of bilobalide before the assays, except in the preliminary experiments where longer incubation times were tested.

Inhibition of Oxygen Consumption

Oxygen consumption was followed by a Clark electrode. Reactions were performed at 25° in a 2.5-mL chamber containing 2 mg of mitochondrial suspension in the incubation buffer. Respiration rates were measured using 10 mM D-β-hydroxybutyrate (for complex I), 5 mM succinate (for complexes III and V), or 6 mM $Fe(CN)_3$ (for complex IV) as substrates. Inhibitor studies were performed by the addition of different concentrations of Amytal (for complex I), antimycin A (for complex III), KCN (for complex IV), or oligomycin (for complex V) to a reaction containing non-limiting amounts of substrate. It must be noted that three different stocks of antimycin A solution in ethanol were used throughout this work.

The experiment was performed as follows. O_2 consumption was measured for mitochondria in the presence of the substrate, the inhibitor was then added to the chamber, and O_2 consumption was again measured. This was done both for control mitochondria and for mitochondria preincubated with bilobalide or isolated from bilobalide-treated rats. The percentage of inhibition was calculated as "100 × (O_2 consumption without inhibitor $-O_2$ consumption with inhibitor)/O_2 consumption without inhibitor."

Activity of Complex I

The activity of NADH cytochrome *c* reductase was measured according to Boffoli *et al.* [27]. Mitochondria were frozen at −70°, thawed, and 23 μL of the suspension was diluted in 1 mL of 25 mM potassium phosphate buffer, 5 mM $MgCl_2$, pH 7.4 containing 10 μM cytochrome *c* and 2.5 mg/mL BSA and sonicated for 30 sec at 4°. KCN (2 mM) was added, the mitochondria incubated for 5 min at 37°, and the reaction initiated by the addition of 2.5 mM NADH. The reduction of cytochrome *c* was followed at 550 nm at 37°. In order to subtract the reduction of cytochrome *c* which was independent of the complex I activity, the same reaction was measured in the presence of rotenone 2 μg/mL.

Adenine Translocase

Adenine nucleotide translocase activity was determined in terms of atractyloside-sensitive ADP uptake using the procedure of Duée and Vignais [28] adapted by Duan and Karmazyn [29]. Briefly, 50 nmol of [^{14}C]-ADP was added to the reaction medium containing 110 mM KCl, 20 mM Tris, and 1 mM EDTA at pH 7.4 and 500 μg of mitochondrial protein. The reaction was carried out on ice and stopped after 60 sec by the addition of 100 μM atractyloside. This reaction time was selected because preliminary results showed that, by this time, ADP was at 50% of the maximum transport in the mitochondria. The reaction mixture was centrifuged at 25,000 *g* for 5 min and the supernatant discarded. The pellet was washed twice with ice-cold reaction medium, dissolved in 0.5 mL of NaOH 0.5 N, and counted for radioactivity in 2.5 mL of scintillation fluid (Aqualuma, Lumac) in liquid scintillation counter.

Statistical Analysis

Results are presented as means ± SD. Statistical analyses were performed using Student's *t*-tests or one-way analysis of variance with Scheffé's contrasts for multiple comparison.

RESULTS

RCR: Optimal Conditions

Bilobalide treatment dose dependently increased the RCR of mitochondria: a significant increase from 8.3 for control mitochondria to 12.1 for mitochondria from rats treated with 4 mg/kg and a highly significant increase from 7.72 to 13.3 for rats treated with 8 mg/kg (Fig. 1A). On the other hand, the P/O ratio, which gives the number of moles of ATP generated per atom of oxygen consumed in respiration, did not change. The P/O ratio values were 3.17 ± 0.18 (N = 9) for control mitochondria and 3.36 ± 0.08, 3.14 ± 0.13, and 2.92 ± 0.19 for mitochondria from rats treated with 2, 4, and 8 mg/kg bilobalide, respectively (N = 3). The bilobalide-induced increase in RCR was the result of a significant decrease in state 4 of 42% and 64% at 4 and 8 mg/kg, respectively (Fig. 1C). State 3 also decreased but to a much lesser extent, with a 8% and 36% decrease in rats treated with 4 and 8 mg/kg bilobalide, respectively (Fig. 1B).

Whether bilobalide could also increase the RCR of control mitochondria isolated from untreated rats was then investigated by incubating these mitochondria directly in the presence of this molecule before the assay. Figure 2A shows that this was indeed the case; the RCR of control mitochondria incubated with 0.08 and 0.8 μg/mL bilobalide increased by 47% and 55%, respectively after 1 hr incubation. This increase in RCR was again the result of a marked decrease in state 4 (Fig. 2B) even though state 3 also decreased, but to a lesser extent (Fig. 2C). No further increase in RCR was observed after 2 and 4 hr incubation although the mitochondria were still very well coupled. This stabilization at longer incubation periods was due to the fact that while the effect of bilobalide on state 4 increased with time, its effect on state 3 also increased, so that the ratio between the two remained constant.

These assays were performed without rotenone or EGTA in the incubation buffer. In order to exclude any side effect of succinate transformed into malate and entering through complex I instead of complex III, the effect of bilobalide was tested in the presence of rotenone at 2.4 μM. Bilobalide at 1 μg/mL was still able to increase the RCR from 6.75 ± 0.7 to 9.1 ± 1.55 (N = 3).

Increase in state 4 is often linked to calcium cycling, which increases proton leak [30]. To exclude any effect of bilobalide on this calcium cycling, 1 mM EGTA was added to the incubation buffer of naive mitochondria incubated for 1 hr in the presence of 1 μg/mL bilobalide. The effect of

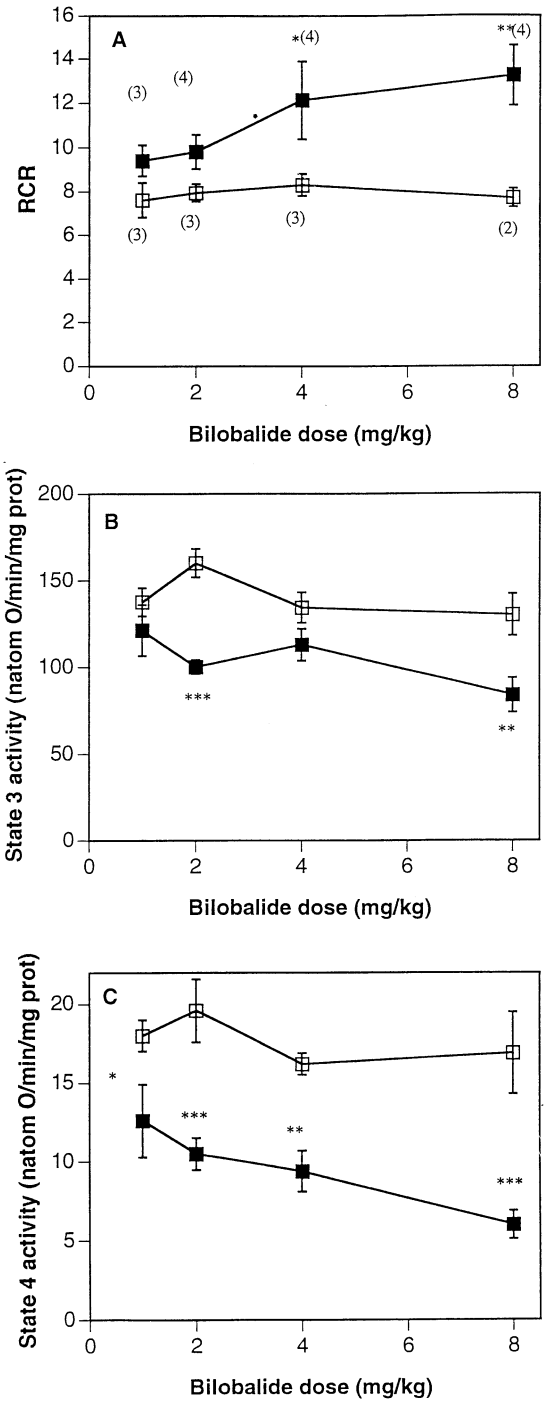

FIG. 1. Effect of bilobalide treatment on mitochondrial respiration. Rats were treated for 14 days with different doses of bilobalide (■) or with distilled water (□). The liver mitochondria were then purified. The control group is divided into 4 different subgroups because one subgroup of control rats corresponds to one subgroup of rats treated with one dose of bilobalide: the rats of both subgroups belong to the same batch, purchased the same day, housed together and killed the same day. State 3 (B) and state 4 (C) of the respiration were measured and RCR was calculated (A). Results are expressed as means ± SD. The number of replicates is indicated in parentheses. *, **, or ***: significantly different from corresponding control with $P < 0.05$, 0.01, or 0.001 using one-way analysis of variance with Scheffé's contrasts.

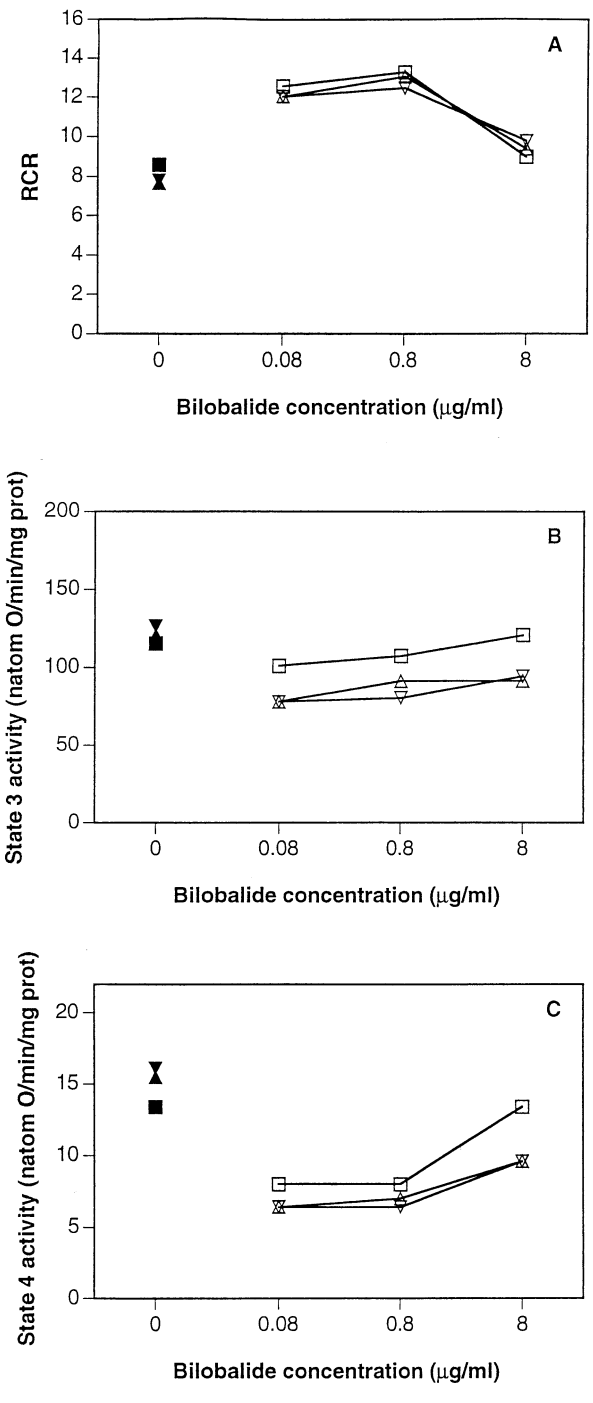

FIG. 2. Effect of bilobalide preincubation on mitochondrial respiration. Liver mitochondria were purified from non-treated rats. Mitochondria were then incubated for 1 (□), 2 (△), or 4 hr (▽) with different concentrations of bilobalide (open symbols) or with distilled water (closed symbols). State 3 (B) and state 4 (C) of the respiration were measured and RCR was calculated (A). Results are expressed as means ± SD for N = 3.

bilobalide was the same in the absence (from 6.75 ± 0.7 to 9.1 ± 1.5; N = 3) or in the presence of EGTA (from 6.25 ± .25 to 9.2 ± 0.6; N = 3). This result indicates that bilobalide did not act either as a calcium chelator or via the inhibition of calcium cycling.

Inhibition of Mitochondrial Complexes

To understand the effect of bilobalide on mitochondrial respiration, evidence for a site-specific effect of bilobalide was sought. A series of inhibitors acting on the different complexes of the respiratory chain were used in order to further delineate the possible site of bilobalide action: Amytal to block the activity of complex I using β-hydroxybutyrate as substrate, antimycin A to inhibit complex III, cyanide for cytochrome *c* oxidase (complex IV), and oligomycin for ATP synthase (complex V), all using succinate as substrate and measuring the inhibition on oxygen consumption. These experiments were performed with liver mitochondria isolated from rats treated with 8 mg/kg bilobalide as well as with naive mitochondria preincubated for 1 hr with 0.8 μg/mL bilobalide.

Amytal dose-dependently inhibited respiratory activity from 18% at 0.1 μM to 80–85% at 0.8 μM. Figure 3A shows that this inhibitory curve was shifted to the right when mitochondria were isolated from bilobalide-treated rats, indicating that bilobalide-treated mitochondria were partly protected from inhibition by Amytal. An inhibition of 50% was obtained with 0.2 μM Amytal for the control mitochondria, while 0.3 μM was necessary to achieve the same inhibition in bilobalide-treated mitochondria. The same protective effect was observed when mitochondria were directly preincubated for 1 hr with 0.8 μg/mL bilobalide (Fig. 3B).

A similar shift in the inhibition curve of O_2 consumption was observed with antimycin A. Bilobalide, either in treatment (Fig. 4A) or during the 1-hr preincubation (Fig. 4B), increased the resistance of mitochondria respiratory activity to inhibition by antimycin A. The concentration of antimycin A needed to inhibit 50% of the O_2 consumption of control mitochondria compared to mitochondria from bilobalide-treated rats shifted from 25 to 36 μM. A maximum 51% protection was observed at 28 nM antimycin A when bilobalide was used in treatment, rising to 85% at 52 nM antimycin A when mitochondria were preincubated with bilobalide. Antimycin A inhibits complex III activity by blocking electron transfer from cytochrome *b* to cytochrome *c1*, while myxothiazol blocks electron transfer from ubiquinol to the Rieske iron sulfur protein, which is located upstream in the electron flow [31]. The effect of bilobalide on myxothiazol inhibition of complex III activity was then investigated. Figure 4C shows that myxothiazol dose dependently inhibited respiratory activity from 11.5% at 0.8 μM to 92% at 7.7 μM, and that preincubation of mitochondria in the presence of 0.8 μM/mL bilobalide significantly decreased the sensitivity of O_2 consumption to this inhibition: a maximum of 33% protection was observed at 3.1 μM myxothiazol. An inhibition of 50% was obtained with 2.4 μM myxothiazol for the control mitochondria, while 4.1 μM was necessary for bilobalide-treated mitochondria to achieve the same inhibition.

The effect of bilobalide on the susceptibility of respiratory activity to KCN and to oligomycin was also examined.

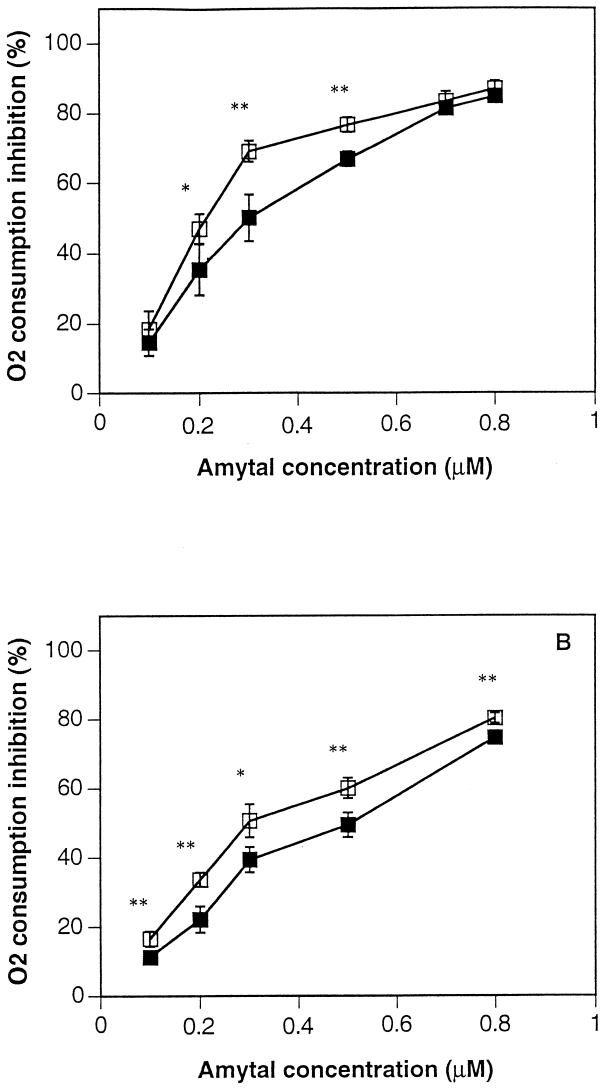

FIG. 3. Effect of bilobalide on the inhibition of complex I. Liver mitochondria were purified either from rats treated for 14 days with 8 mg/kg bilobalide (■) or distilled water (□) (A) or from non-treated rats preincubated for 1 hr with (■) or without (□) 0.8 μg/mL bilobalide (B). O_2 consumption was then measured in the absence and presence of different concentrations of Amytal. Initial RCR values were (A) 5.75 and 6.72 and (B) 7.4 and 10.8 for control mitochondria and bilobalide mitochondria, respectively. Results are expressed in percentage of inhibition of RCR compared to measurement without Amytal as means ± SD (N = 3). * or **: significantly different from corresponding control with $P < 0.05$ or 0.01 using Student's *t*-test.

Bilobalide neither in treatment nor in preincubation affected the inhibition of O_2 consumption due to cytochrome *c* oxidase inhibition by KCN (Fig. 5) or to ATP synthase inhibition by oligomycin (Fig. 6). The results of these studies suggest that bilobalide interacts with complexes I and III but not with complexes IV or V.

A non-specific effect of bilobalide on mitochondria membrane permeability is also possible. This effect was assessed when mCCP was used to uncouple mitochondria by dissipating the proton gradient. Figure 7 shows that

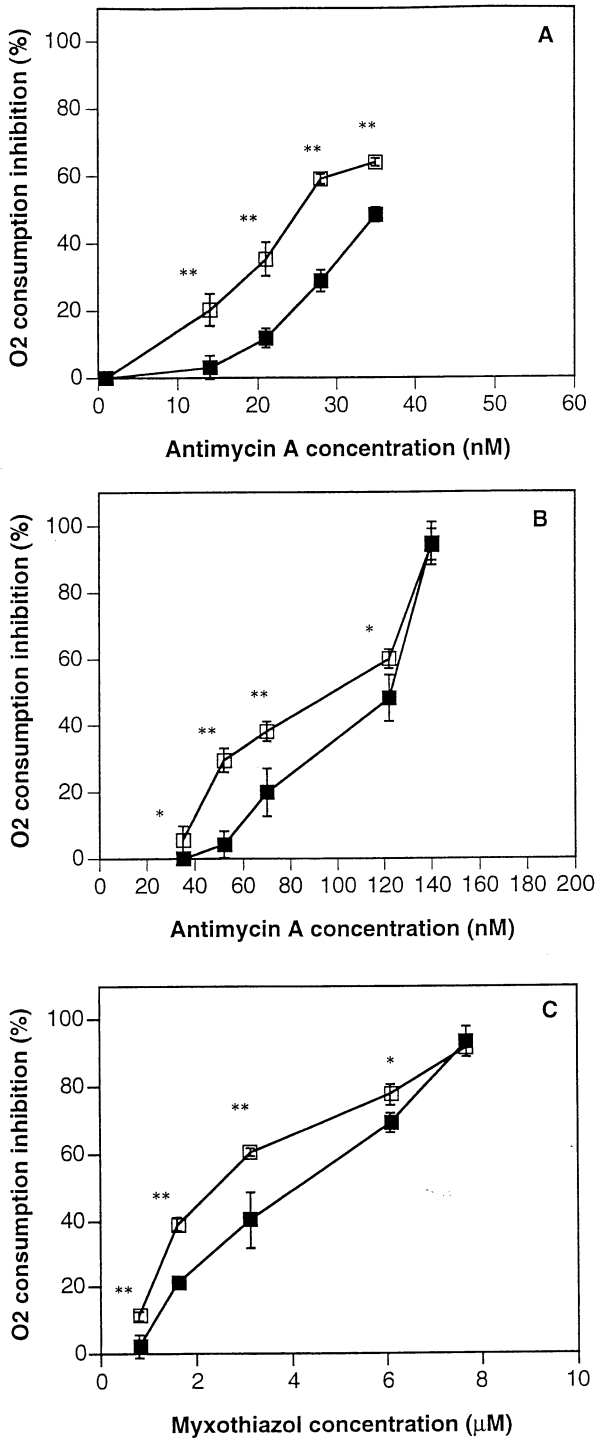

FIG. 4. Effect of bilobalide on the inhibition of complex III. Liver mitochondria were purified either from rats treated 14 days with 8 mg/kg bilobalide (■) or distilled water (□) (A) or from non-treated rats preincubated for 1 hr with (■) or without (□) 0.8 μg/mL bilobalide (B and C). O_2 consumption was then measured in the absence and presence of different concentrations of antimycin A (A and B) or myxothiazol (C). Initial RCR values were (A) 4.42 and 5.2 and (B) 6.4 and 8.2 for control mitochondria and bilobalide mitochondria, respectively. Results are expressed in percentage of inhibition of RCR compared to measurement without inhibitor as means ± SD (N = 3). * or **: significantly different from corresponding control with $P < 0.05$ or 0.01 using Student's *t*-test.

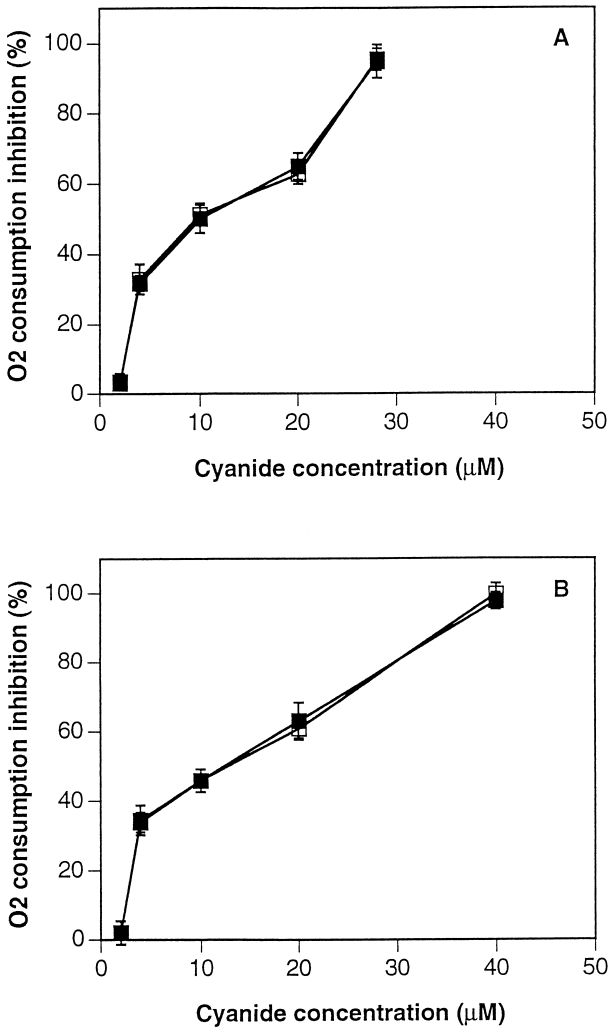

FIG. 5. Effect of bilobalide on the inhibition of complex IV. Liver mitochondria were purified either from rats treated for 14 days with 8 mg/kg bilobalide (■) or distilled water (□) (A) or from non-treated rats preincubated for 1 hr with (■) or without (□) 0.8 μg/mL bilobalide (B). O_2 consumption was then measured in the absence and presence of different concentrations of KCN. Initial RCR values were (A) 6.2 and 7.8 and (B) 7.1 and 9.65 for control mitochondria and bilobalide mitochondria, respectively. Results are expressed in percentage of inhibition of RCR compared to measurement without KCN as means ± SD (N = 3).

bilobalide, neither in treatment nor in preincubation, could change the sensitivity of RCR to inhibition by mCCP, indicating that bilobalide does not act on the fluidity of the inner mitochondrial membrane.

Effect of Bilobalide on the Different Parameters of Mitochondrial Respiratory Activity

The studies of the effect of bilobalide in the presence of the various inhibitors were performed separately on mitochondria purified from control or treated rats. Bilobalide at 8 mg/kg was shown to increase the RCR by decreasing state 4 and to protect complexes I and III from inhibition. In order to

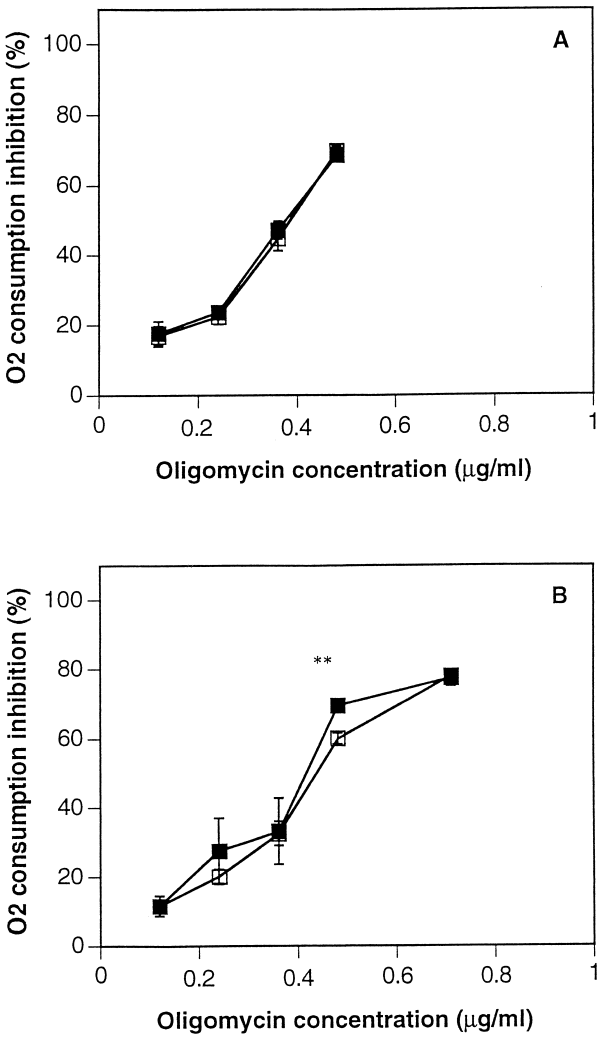

FIG. 6. Effect of bilobalide on the inhibition of complex V. Liver mitochondria were purified either from rats treated for 14 days with 8 mg/kg bilobalide (■) or distilled water (□) (A) or from non-treated rats preincubated for 1 hr with (■) or without (□) 0.8 μg/mL bilobalide (B). O_2 consumption was then measured in the absence and presence of different concentrations of oligomycin. Initial RCR values were (A) 6.31 and 7.68 and (B) 6.12 and 8.16 for control mitochondria and bilobalide mitochondria, respectively. Results are expressed in percentage of inhibition of RCR compared to measurement without oligomycin as means ± SD (N = 3). **: significantly different from corresponding control with $P < 0.01$ using Student's *t*-test.

confirm these results and to limit the variability, all the assays were then performed on the same mitochondrial preparations obtained from rats treated with two doses of bilobalide.

For this purpose, three rats each were treated with 2 mg/kg bilobalide, 8 mg/kg bilobalide, with distilled water for controls. Liver mitochondria were isolated from each rat, and the RCR was measured using complex II substrate succinate and complex I substrates glutamate/malate. The inhibitory effects of Amytal and antimycin were also assessed as well as the real activity of complex I and adenine translocase. All the results are summarized in Table 1. Analysis of the results confirmed a significant 21% increase in RCR for the mitochondria from rats treated with 8 mg/kg bilobalide compared to control mitochondria. This increase was due to a decrease in state 4 both when succinate (61%) and glutamate/malate (68%) were used as substrates. The protective effect of bilobalide on complex I and III inhibition was also confirmed, but the effect on complex III was more pronounced since a complete protection was obtained in the presence of 560 nM antimycin A compared to 32% inhibition for the control mitochondria. Moreover, the activity of complex I was significantly increased in mito-

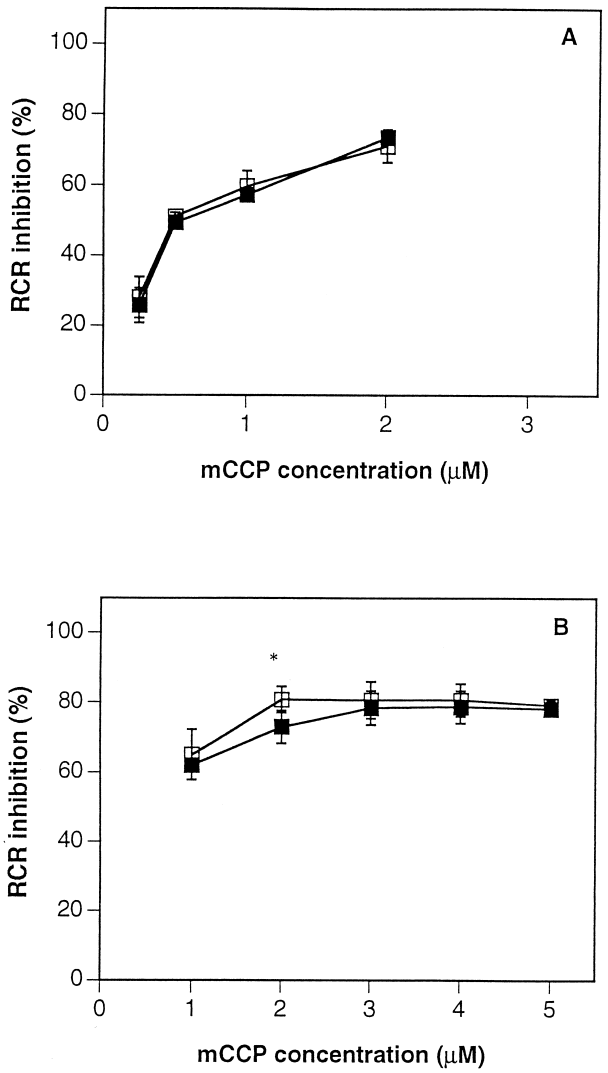

FIG. 7. Effect of bilobalide on uncoupling by mCCP. Liver mitochondria were purified either from rats treated for 14 days with 8 mg/kg bilobalide (■) or distilled water (□) (A) or from non-treated rats preincubated for 1 hr with (■) or without (□) 0.8 μg/mL bilobalide (B). RCR was then measured in the absence and presence of different concentrations of mCCP. Initial RCR values were (A) 4.2 and 5.43 and (B) 7.54 and 10.28 for control mitochondria and bilobalide mitochondria, respectively. Results are expressed in percentage of inhibition of RCR compared to measurement without mCCP as means ± SD (N = 3). *: significantly different from corresponding control with $P < 0.05$ using Student's *t*-test.

TABLE 1. Effect of bilobalide on the respiratory activity of treated rats

	Control	Bilobalide 2 mg/kg	Bilobalide 8 mg/kg
RCR (succinate)	5.8 ± 0.4	6.06 ± 0.05	7.02 ± 0.47†
State 4	13.7 ± 2.3	12.3 ± 2.3	8.3 ± 1.6*
State 3	79.98 ± 18.2	68.4 ± 13.6	58.7 ± 13.2
RCR (glutamate/malate)	7.72 ± 0.58	7.78 ± 0.56	9.26 ± 0.54*
State 4	6.3 ± 1.3	5.8 ± 1.8	4.3 ± 1.2
State 3	48.8 ± 10.6	44.3 ± 12.1	38.8 ± 9.9
P/O ratio	2.1 ± 0.27	2.13 ± 0.15	2.1 ± 0.73
Inhibition of complex I (at 0.4 μM Amytal)	36.3 ± 5.8%	21.9 ± 8.5%*	25.0 ± 2.6%
Inhibition of complex III (at 560 nM antimycin A)	32.2 ± 6.8%	11.2 ± 5.4%	1.3 ± 1.8%†
Activity of complex I	0.46 ± 0.08	0.46 ± 0.08	0.73 ± 0.06*
Activity of adenine translocase (at 60 sec)	10,668 ± 595	ND	9,739 ± 2,543

Rats were treated for 14 days with 2 or 8 mg/kg bilobalide or with distilled water. The liver mitochondria were then purified. State 3 and state 4 of the respiration were measured in the presence of succinate or glutamate/malate as substrates and the RCR was calculated. The same mitochondrial fractions were used to assay for the inhibition of complex I by Amytal and of complex III by antimycin A as well as the activity of complex I and of adenine translocase. Results are expressed in natom O/min/mg proteins for states 3 and 4, in percentage of O_2 consumption inhibition in the presence of Amytal or antimycin A, in U/mg proteins for complex I activity, and in dpm of [^{14}C]-ADP incorporation into mitochondria for adenine translocase activity. Results are presented as means ± SD for N = 4 for control and N = 3 for bilobalide 2 and 8 mg/kg. ND: not determined.

* or †: significantly different from corresponding control with $P < 0.05$ or $P < 0.01$ using one-way analysis of variance with Scheffé's contrasts.

chondria isolated from bilobalide-treated rats, reaching 159% of the control activity at 8 mg/kg bilobalide. These results clearly show a strong correlation between complex I and III protection by bilobalide and its influence on state 4 and thus on the RCR. Similar protective effects were also obtained on mitochondria isolated from rats treated with 2 mg/kg bilobalide, but these effects did not reach statistical significance. Again, the most marked effect was observed on the protection of complex III inhibition by antimycin A.

Bilobalide treatment (2 or 8 mg/kg) did not change adenine translocase activity (Table 1), nor did bilobalide (0.8 μg/mL) in preincubation in two separate experiments: [^{14}C]-ADP incorporation for 40 sec led to 13,103 ± 296 and 15,593 ± 344 dpm for control mitochondria compared to 13,874 ± 755 and 17,098 ± 514 for bilobalide-preincubated mitochondria. The maximal incorporation of ADP after 10 min was 26,150 dpm.

Liver Ischemia

These experiments showed that bilobalide increased respiratory activity when this activity was measured in mitochondria kept under optimal conditions. In order to estimate the effect of bilobalide under ischemic conditions, livers from rats treated with 8 mg/kg bilobalide or with distilled water were subjected to 10-min normoxic perfusion or to 10-min ischemia. After isolation of the mitochondria, states 3 and 4 of respiration were measured and the RCR calculated. Table 2 shows that the RCR of mitochondria isolated from normoxia-perfused liver was lower than that of mitochondria from unperfused liver, indicating that the perfusion per se induced damage to the mitochondria. In both mitochondria preparations, bilobalide significantly increased RCR; however, contrary to what was observed for unperfused liver mitochondria, this increase was mainly due to a large increase in state 3. Ischemia significantly decreased RCR compared to normoxic perfusion from 4.8 to 3.7. A decrease in state 3 accounted for this lower RCR. State 4 in mitochondria from livers submitted to ischemia remained at the same level as that measured in mitochondria maintained under normoxic conditions. Bilobalide completely protected this

TABLE 2. Effect of bilobalide on the respiratory activity after liver ischemia

	Control	Bilobalide
0 min		
State 3	130.2 ± 13.1	83.9 ± 9.9
State 4	16.9 ± 2.6	6.0 ± 0.9
RCR	7.72 ± 0.43	13.25 ± 1.36
P/O ratio	2.97 ± 0.15	2.92 ± 0.19
10 min normoxic perfusion		
State 3	110.8	141.6
State 4	22.2	25.3
RCR	4.8	5.22
P/O ratio	3.05	2.94
10 min ischemia		
State 3	89.3 ± 4.5	139.9 ± 19.6
State 4	23.9 ± 1.2	24.6 ± 1.6
RCR	3.7 ± 0.5	5.2 ± 0.6
P/O ratio	1.99 ± 0.29	2.54 ± 0.41

Rats were treated for 14 days with 8 mg/kg bilobalide or with distilled water (controls). The liver mitochondria were then purified either directly after killing (0 min) or after 10 min of normoxic perfusion or 10 min clamping (ischemia). State 3 and state 4 of the respiration were measured in the presence of succinate as substrate and the RCR was calculated. Results are expressed in natom O/min/mg proteins for states 3 and 4 and presented as means ± SD for N = 3 or as means for N = 2 for normoxia.

ischemia-induced decrease in state 3, and the RCR of these mitochondria was identical to that of mitochondria isolated from normoxia-perfused liver.

DISCUSSION

The protective effects of the *Ginkgo biloba* extract against deterioration of metabolism and function caused by hypoxia in brain [14–16] and in heart have previously been demonstrated in rats [18]. This protective effect has been attributable to the terpenoid fraction of EGb 761 and at least in part to bilobalide. The anti-ischemic activity of EGb 761 has also been observed in double-blind clinical studies, for example in the treatment of peripheral arterial occlusive disease [32, 33], and in patients with cerebrovascular insufficiency in old age [34].

Recent findings appear useful in explaining the anti-ischemic activity of the terpenoid constituents of EGb 761. Janssens *et al.* [24] showed that bilobalide prevented a hypoxia-induced decrease in ATP in cultured endothelial cells by protecting mitochondrial coupling. Sparing of high energy phosphates, such as ATP, during ischemia could preserve cellular integrity and functions during ischemia but could also prevent reactive oxygen species formation during reperfusion, thereby favoring functional recovery.

The present study aimed to define the mechanism whereby bilobalide maintains mitochondrial coupling. Mitochondria isolated from bilobalide-treated (8 mg/kg) rats as well as control mitochondria preincubated for 1 hr with 0.8 μM bilobalide showed a markedly increased RCR which was due to a decrease in state 4 of the respiration. A dose-dependent effect was also observed. The lower effect at 8 μg/mL bilobalide is probably due to a loss of effect of this molecule at higher concentrations, as observed earlier [24]. State 4 reflects the proton leak across the inner mitochondrial membrane. At least two processes can influence the level of such a leak. First, state 4 mainly depends on the optimal activity and integrity of the respiratory chain, namely of the different electron transporters (complexes I, III, and IV) which extrude protons from the mitochondrial matrix toward the intermembrane space, thus generating the proton gradient [35]. Secondly, when cytosolic calcium concentration rises and/or when mitochondria are slightly damaged, calcium cycling takes place, dissipating the proton gradient and increasing state 4. This was prevented by calcium chelators such as EGTA [30]. The results presented herein show that the effect of bilobalide in the presence of EGTA was the same as in the absence of EGTA, thus excluding a role for bilobalide as calcium chelator or as inhibitor of calcium cycling. On the other hand, bilobalide was able to protect the respiratory chain from inhibition of complex I activity by Amytal as well of complex III activity by antimycin A or myxothiazol. Bilobalide treatment was also able to increase the complex I activity of liver mitochondria. These results show that bilobalide increases the activity of complex I and protects both complexes I and III from inhibition and hence, leads to a decrease in state 4. Interestingly, EGb 761 as well as bilobalide have also been shown to be protective against cerebral edema induced by triethyltin, which uncouples oxidative phosphorylation [36].

Bilobalide also slightly decreased state 3 in native mitochondria. Under optimal conditions, state 3 is mainly limited by the activity of adenine translocase [29]. However, bilobalide did not affect adenine translocase activity either in treatment or in preincubation. Succinate transport is also important for state 3 activity [37, 38], but bilobalide did not seem to influence this parameter since a similar effect of bilobalide on state 3 was observed when succinate or glutamate/malate was used as substrate. The most important effect of bilobalide for mitochondria under optimal conditions is to decrease state 4. The P/O ratio is not affected by bilobalide treatment of preincubation, which means that the same amount of O_2 is needed to produce a given amount of ATP with or without bilobalide. If the mitochondria are better coupled in the presence of bilobalide (state 4 is decreased), a high rate state 3 may not be needed to obtain the same efficiency in terms of ATP regeneration. This could explain why state 3 is decreased in the presence of bilobalide.

Most interestingly, bilobalide totally protects the ischemia-induced decrease in RCR. Ischemia decreases state 3 and bilobalide completely prevents this decrease. The question is asked as to why a protection of state 3 is observed for ischemic mitochondria in the presence of bilobalide while there is no protection in normal mitochondria. The following explanation is proposed: state 3 determination is mainly limited by adenine translocase activity in normal mitochondria, while complexes I and III function at approximately 70% of their maximal activity. However, when alterations of the mitochondrial chain occur, the activity of the different complexes begins to be the limiting factor so that state 3 decreases and the RCR is lower. Since bilobalide acts on complexes I and III but not on adenine translocase, it is clear why no effect of bilobalide can be observed on state 3 when the respiratory chain is functioning optimally (i.e. for mitochondria isolated from unperfused liver). On the other hand, when there are small alterations, then the activity of the complexes is limiting and the effect of bilobalide that increases the activity of such complexes can then be observed on state 3 (i.e. for mitochondria isolated for normoxia-perfused liver). The effect of bilobalide is even more pronounced when state 3 is decreased to a larger extent, for example after ischemia.

The exact mechanism whereby bilobalide operates on complexes I and III is not known. Several hypotheses can be put forward: 1) Bilobalide could act as an antioxidant scavenging superoxide anion generated by electron leakage. This leakage occurs mainly at the level of ubisemiquinone and increases during ischemia/reperfusion [39]. Ubiquinol is the electron transporter between complexes I and III. However, bilobalide is not able to scavenge superoxide anion or hydroxyl radical *in vitro* [18, 40]; 2) Bilobalide could also scavenge NO (nitric oxide). However, the

enzyme of mitochondrial respiration which is inhibited by NO is cytochrome *c* oxidase [41]. This enzyme does not seem to be a target for bilobalide; and 3) Bilobalide could be by itself an electron transporter, thus increasing electron transfer from complex I to complex III. No evidence which could sustain such a mechanism is currently available. In order to observe the effect of bilobalide, bilobalide must accumulate or insert itself into the inner mitochondrial membrane, since no effect of bilobalide can be observed if the treatment is shorter than 12 days [24] or if the preincubation is less than 30 min (data not shown). These observations suggest that bilobalide does not act as a substrate or as some drugs such as ifenprodil or vincamine which are able to increase RCR when added directly to the mitochondrial suspension [42]. The nature of the alteration responsible for the decrease in complex I and III activity during ischemia is not known, nor is the mechanism of bilobalide protection. Both need further investigation.

Ischemic conditions have been associated with functional and morphological defects in mitochondria: inhibition of the respiratory chain and an increase in the proton leak have been observed [43]. A decrease in complex I activity [6–8] as well as in adenine translocase activity [29] seem to be mainly responsible for the decrease in oxidative phosphorylations. Alteration of complex III activity also occurs to a lesser extent or much later [7, 8].

By maintaining a high respiratory chain activity, bilobalide could protect mitochondrial functional impairment induced by ischemia, thus allowing ATP regeneration to continue under ischemic conditions. Preservation of the ATP pool under such conditions could preserve cellular functions and prevent tissue ischemia-induced damage. This mechanism provides a possible explanation for the anti-ischemic properties of bilobalide and of EGb in therapeutic interventions.

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