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Effects of *Ginkgo biloba* extract on heart and liver mitochondrial functions: mechanism(s) of action

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Abstract Though extracts of *Ginkgo biloba* leaves (GBE) have a wide pharmacological application, little is known about GBE effects on mitochondria. In this work, effects of ethanolic GBE on the respiration of isolated rat heart and liver mitochondria were investigated. We found that GBE stimulates the pyruvate + malate-dependent State 2 respiration of heart mitochondria and decreases mitochondrial membrane potential. Uncoupling effect of GBE was found to be due to its protonophoric action and is likely to be mediated by the ATP/ADP-translocator and uncoupling proteins. The effect of GBE was less in liver than in heart mitochondria. State 3 respiration of heart mitochondria was slightly stimulated at low and depressed at higher GBE concentrations. Inhibition of State 3 respiration of heart mitochondria was not relieved by uncoupler indicating that GBE may inhibit the respiratory chain complexes or the substrate transport. However, Complex IV of the respiratory chain was not inhibited by GBE. H_2O_2 generation was attenuated by low concentration of GBE probably due to mild uncoupling. The data suggest that mild but not severe uncoupling activity of GBE may be important in providing pharmacological protection of cellular functions in pathological situations.

Keywords *Ginkgo biloba* extract (GBE) · Mitochondria · Permeabilized fibers · Respiration · Uncoupling of oxidative phosphorylation

Abbreviations

ANT	Adenine nucleotide translocase
CAT	Carboxyatractiloside
CCCP	Carbonylcyanide p-chloromethoxyphenylhydrazone
ROS	Reactive oxygen species
UCP	Uncoupling protein
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
TPP^+	Tetraphenylphosphonium

Introduction

Ginkgo biloba L. (*Ginkgoaceae*), originated from China, first introduced to Europe in the 18th century (Stromgaard and Nakanishi 2004), is now distributed all over the world. Over the past 20 years, *Ginkgo biloba*-derived preparations have become widely used in medical practice. A wide pharmacological application of *Ginkgo biloba* extracts (GBE) is determined by the main active substances: flavonoids (flavone glycosides, primarily composed of quercetin) and terpenoids (ginkolides and bilobalides). Due to their vasorelaxing, anticoagulant, antioxidative and anti-inflammatory properties, GBE have been most frequently prescribed as preparations that improve cerebral blood circulation and memory (Christen 2004; Luo 2001).

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Clinical trials and studies on experimental animals showed that EGb 761, a standardized dry extract of *Ginkgo biloba* leaves, consisting of 24% ginkgo flavone glycosides and 6% terpenoids, improves myocardial functional recovery and reduces the incidence of ventricular arrhythmias induced by ischemia-reperfusion (Clostre 2001; Varga et al. 1999; Varga 2002; Shen et al. 1998). Beneficial action of EGb 761 was related to inhibition of reactive oxygen species (ROS) and NO production in the ischemic/reperfused myocardium (Pietri et al. 1997; Varga et al. 1999). It is postulated that both flavonoid and terpenoid constituents are involved in the antioxidant effects of GBE (DeFeudis and Drieu 2000; Akhlaghi and Bandy 2009).

Findings of different authors revealed that EGb 761 has multiple effects on mitochondrial functions and apoptotic pathways: stabilization of mitochondrial membrane potential and mitochondrial respiratory chain function, improvement of energy metabolism, upregulation of anti-apoptotic Bcl-2 protein and downregulation of pro-apoptotic Bax, inhibition of cytochrome c release from mitochondria, reduction of caspase 9 and caspase 3 activity after oxidative stress, and reduction of apoptotic cell death (Eckert et al. 2005; Tendi et al. 2002). However, it should be noted that (1) in most investigations, PC12 cells, isolated brain cells and isolated brain mitochondria were used, and (2) little or no attention was paid to assess the GBE effects on the mitochondria of other tissues (heart, liver, etc), activities of their enzyme systems and the mechanisms of action.

In our previous study confined to very low GBE concentrations, we revealed a very powerful uncoupling effect of GBE in ethanol, i.e., the dose-dependent stimulation of the State 2 respiration of rat heart mitochondria (Trumbeckaite et al. 2007). Interestingly, the pure flavonoids—rutin, quercetin and quercitrin (except of hyperosid)—at concentration identical to that in GBE, and their mixture stimulated the State 2 respiration far less than GBE. Obviously, the much stronger GBE-induced uncoupling of oxidative phosphorylation may be determined by other, even minor, compounds of GBE, or by their synergistic action (Wagner and Ulrich-Merzenich 2009).

Many of GBE compounds are polyphenol substances, weak acids of hydrophobic character and thus may possess protonophoric properties similar to classical uncouplers of oxidative phosphorylation. It is also known (Parker et al. 2008; Harper et al. 2008) that proton conductance can be mediated by ANT and by UCPs. However, these possible mechanism(s) for GBE-induced uncoupling of mitochondrial oxidative phosphorylation (activation of proton conductance in rat heart mitochondria via UCPs, ANT or due to protonophoric action) were not investigated so far and, therefore, need clarification.

The aims of this work were: (1) to investigate and compare the concentration-dependent GBE effects on the respiration of isolated rat heart and liver mitochondria and permeabilized rat heart fibers, (2) to investigate the mechanism(s) of GBE-induced uncoupling and its relevance to ROS production in rat heart mitochondria.

Materials and methods

Materials

Amplex Red was purchased from Invitrogen. All other reagents were from Sigma/Aldrich and Fluka. Ethanol extract of *Ginkgo biloba* leaves, "Ginkomed", was from "Valentis", Vilnius, Lithuania (the flavonoids identified by HPLC method are indicated previously, Trumbeckaite et al. 2007).

Preparation of rat heart and liver mitochondria

All experimental procedures were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Licence No. 0006). Hearts and livers from 2–4-month-old male Wistar rats were excised and rinsed in ice-cold 0.9% KCl solution. The tissues were cut into small pieces and homogenized in a glass-Teflon homogenizer in a medium containing 180 mM KCl, 10 mM Tris/HCl, 5 mM EGTA, and 1 mg/ml bovine serum albumin (BSA) (pH 7.7) for heart or in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 5 mg/ml BSA, pH 7.4 for liver. The heart homogenate was centrifuged 5 min at 750×*g*, and the supernatant was centrifuged at 6,300×*g* for 10 min. The mitochondrial pellet was re-suspended in the buffer containing 180 mM KCl, 20 mM Tris/HCl (pH 7.35) to approximately 50 mg/ml protein, and stored on ice. The liver homogenate was centrifuged at 750×*g* for 5 min, and the supernatant was centrifuged at 7,000×*g* for 10 min. The pellet was washed in a buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4. The final centrifugation was done at 7,000×*g* for 10 min. The mitochondrial pellet was re-suspended in the buffer to an approximate protein concentration of 50 mg/ml. The protein concentration was determined by the biuret method using bovine serum albumin as a standard (Gornal et al. 1949).

Preparation of permeabilized rat heart muscle fibers

Bundles of the rat heart muscle fibers, approximately 0.2–0.3 mm in diameter, were prepared as described (Liobikas et al. 2001; Toleikis et al. 2005). Bundles of heart fibers were transferred to cooled (4°C) solution A containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol,

7.1 mM $MgCl_2$, 50 mM 2-[N-Morpholino]ethanesulfonic acid (MES), 5 mM ATP, 15 mM phosphocreatine, 2.6 mM CaK_2EGTA and 7.4 mM K_2EGTA (free Ca^{2+} concentration 0.1 μM) (pH 7.0 adjusted with KOH at 2°C), supplemented with 50 μg/ml saponin (from Gypsophila; sapogenin content 17%; Sigma) and 3 mg/ml collagenase (Type IV, Sigma) and incubated for 30 min. Then the bundles were washed for 10 min in solution B, containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4, 3.0 mM CaK_2EGTA and 7.1 mM K_2EGTA (free Ca^{2+} concentration 0.1 μM) (pH 7.1 adjusted with KOH at 37°C).

Measurements of mitochondrial respiratory rates

Respiration of isolated rat heart and liver mitochondria was measured at 37°C using a Clark-type electrode in a solution B (see above). Respiratory substrates were 6 mM pyruvate + 6 mM malate, or 10 mM ascorbate + 0.5 mM TMPD in the presence of antimycin A. In some experiments (where indicated), cytochrome c (28 μM) was added. Oxygen uptake rates in permeabilized cardiac fibers were measured in solution B (see above). The State 2 respiration was recorded in the medium containing respiratory substrates but devoid of ADP whereas State 3 respiration was recorded in the medium with respiratory substrates and 2 mM ADP. The solubility of oxygen was estimated to be 422 nmol O/ml. Mitochondrial respiration rates were expressed as nmol O/min/mg protein. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml. Respiratory rates of fibers were expressed as nmol O/min/mg fibers dry $weight^{-1}$ (dry weight = wet weight before respiration measurement/4.85) (Liobikas et al. 2001).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured as described (Borutaite et al. 1995) in a closed, stirred and thermostatically controlled 1.5-ml vessel fitted with a tetraphenylphosphonium (TPP^+)-selective electrode. Mitochondria were incubated in the same medium as for mitochondrial respiration. The experiments were performed at 37°C using 6 mM pyruvate + 6 mM malate as substrate, and 0.5 mg/ml mitochondria.

Measurement of mitochondrial swelling

Swelling of non-respiring rat heart mitochondria was recorded as the decrease of light scattering at 540 nm with the Hitachi 557 spectrophotometer in a potassium acetate medium (120 mM potassium acetate, 10 mM Tris-HCl, 0.5 mM EGTA; pH 7.4 with Trizma base, 25°C) supplemented with 2 μM rotenone and 0.5 μM valinomycin (Nicholls and Ferguson 1992; Kopustinskiene et al. 2002)

Measurement of H_2O_2 generation

H_2O_2 generation was estimated fluorimetrically with a Thermo Scientific platereader. Mitochondria (0.25 mg/ml) were incubated in the same medium B as for mitochondrial respiration (at 37°C) supplemented with 6 mM pyruvate plus 6 mM malate, 0.016 μg/ml antimycin A, 5 μM Amplex Red and 2 U/ml horseradish peroxidase (excitation at 544 nm, emission at 590 nm). Fluorescence signal was calibrated using known amounts of H_2O_2.

Statistical analysis

Data are presented as means±SEM. Each point for every individual preparation averaged for two-three repetitive runs. Statistical analysis was performed using Student's *t* test, and $P<0.05$ was taken as the level of significance. Statistical analysis was performed using the software package Sigma Plot 2000.

Results

In our previous studies (Trumbeckaite et al. 2006, 2007), we observed the powerful uncoupling effect of GBE and some of its flavonoids (quercetin, rutin, hyperoside) on isolated rat heart mitochondria. The similar ~2-fold stimulation of State 2 (in the absence of ADP) respiration by diluted (1:200) GBE (0.5–5 μl/ml) was demonstrated on permeabilized rat heart fibers (Fig. 1a) which contain intact mitochondria and all other intracellular membrane systems interacting structurally and functionally (Saks et al. 1998, 2001). Noteworthy, the similar increase in State 2 respiration rate with pyruvate + malate after addition of GBE (5 μl/ml of diluted 1:200) to isolated rat heart mitochondria was observed in the presence and absence of oligomycin (2.86±0.14 times and 2.79±0.35 times increase, respectively, $n=4$), suggesting that effects of GBE are not related to its action on ATPase.

The uncoupling effect of GBE was also confirmed in the experiments where heart mitochondrial respiratory rate in State 2 was measured simultaneously with the membrane potential. As it can be seen in Fig. 2, GBE at low concentration (2–6 μl/ml, 1:200 dilution) significantly reduced $\Delta\Psi$ of rat heart mitochondria by 4–13 mV and increased the respiratory rate by 21–64 nmol O/min/mg protein.

In contrast to heart mitochondria, the same low concentrations of diluted GBE were not effective in the case of isolated rat liver mitochondria: State 2 respiratory rate was

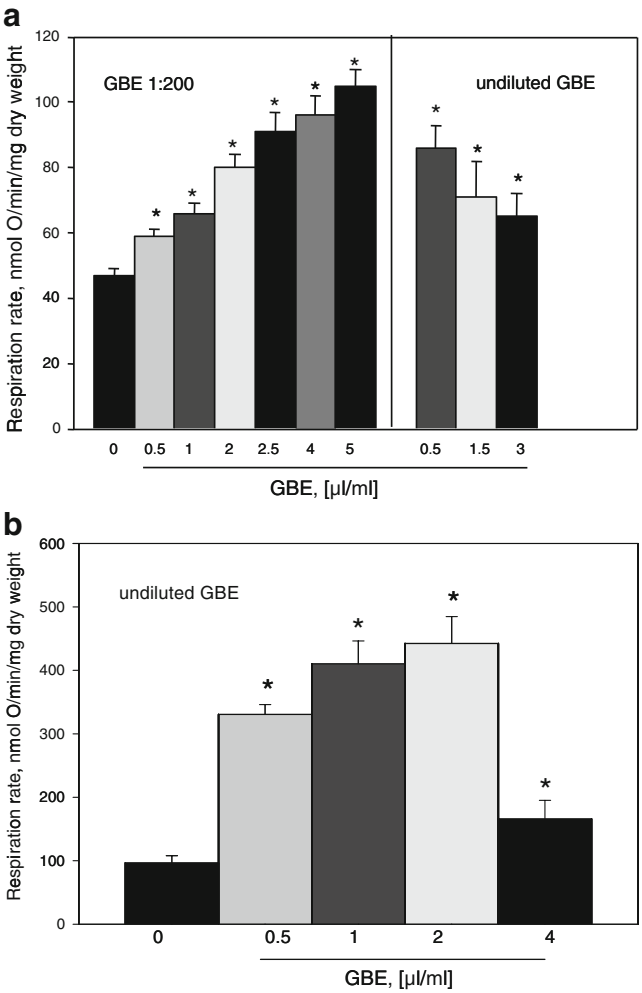

Fig. 1 Effect of *Ginkgo biloba* extract on the mitochondrial State 2 respiration in permeabilized rat heart fibers (**a**) and isolated rat heart mitochondria (**b**). Measurements were performed in the presence of 6 mM pyruvate + 6 mM malate as substrates. In permeabilized heart fibers (**a**) State 2 respiration rate 44±3 nmol O/min/mg protein, State 3 respiration rate 113±10 nmol O/min/mg protein, respiratory control index (RCI) 2.64±0.14; in isolated heart mitochondria (**b**) State 2 respiration rate 96±9 nmol O/min/mg protein, State 3 respiration rate 412±56 nmol O/min/mg protein, RCI 4.5±0.4. Standard incubation medium was used. *$p<0.05$ vs. Control, $n=5–6$

44±4 and 40±5 nmol O/min/mg protein, without and with 4 µl/ml of GBE, respectively; $n=3$) and almost 200 times higher GBE concentrations were needed for liver mitochondria to achieve the effect on the State 2 respiration comparable to heart mitochondria (Fig. 3).

As heart mitochondria are more sensitive to GBE than liver mitochondria, further experiments were performed on rat heart mitochondria. We have previously shown that State 3 (in the presence of ADP) respiration of isolated rat heart mitochondria was not sensitive to low concentrations of GBE (0.5−4 µl/ml, extract diluted 1:200) (Trumbeckaite et al. 2007). However, when heart mitochondria were treated with undiluted GBE, the uncoupling of mitochondrial oxidative

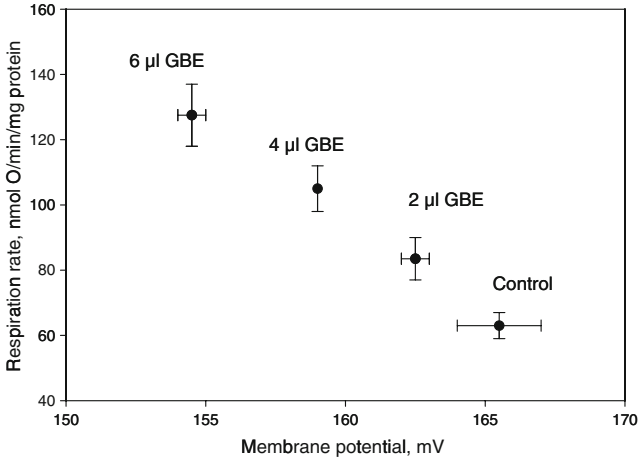

Fig. 2 Effect of diluted (1:200) *Ginkgo biloba* extract on the relationship between membrane potential and respiration rate of isolated rat heart mitochondria in State 2. $n=2$.

phosphorylation was observed: after addition of 0.5–2 µl of undiluted GBE, the State 2 respiration rate with pyruvate + malate increased by 3–4.6 fold (Fig. 1b) achieving the level of State 3 respiration rate (Fig. 4). Noteworthy, these concentrations of GBE also slightly increased the State 3 respiration rate: maximal statistically significant 29% increase was achieved at 1 µl/ml of GBE (Fig. 4). However, further GBE addition notably depressed both the State 2 and State 3 respiration of rat heart mitochondria; the latter decreased by 51% and 81% below control values at 4 µl and 6 µl of GBE, respectively. The State 3 respiration was not restored by CCCP (data not shown, $n=5$) indicating that higher concentrations of GBE may inhibit the activity of respiratory chain complexes or substrate transporters.

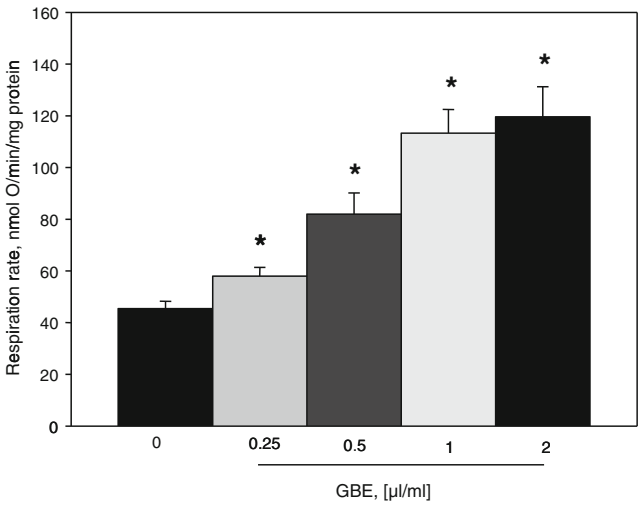

Fig. 3 Effect of undiluted *Ginkgo biloba* extract on respiration of isolated rat liver mitochondria in State 2. Measurements were performed in the presence of 6 mM pyruvate + 6 mM malate as substrates. Standard incubation medium was used. *$P<0.05$ vs. Control, $n=6$

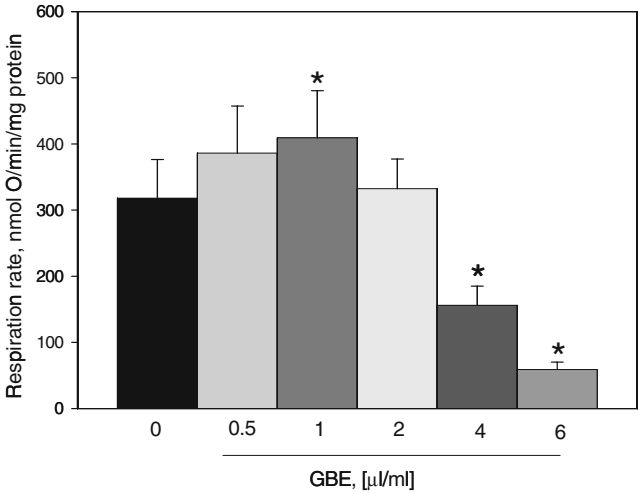

Fig. 4 Effect of undiluted *Ginkgo biloba* extract on respiration of isolated rat heart mitochondria in State 3. Measurements were performed in the presence of 6 mM pyruvate + 6 mM malate as substrates and 2 mM ADP. Standard incubation medium was used. *$P<0.05$ vs. Control, $n=6$

Measurement of Complex IV (cytochrome c oxidase) activity in isolated rat heart mitochondria by following TMPD and ascorbate oxidation (in the presence of antimycin and cytochrome *c*) showed no signs of Complex IV inhibition by undiluted GBE in the range of concentrations of 0.2–4 µl/ml. The Complex IV activity at 2 µl/ml of GBE (1635±78 nmol O/min/mg protein) was not statistically different from respective control (1293± 89 nmol O/min/mg protein) and the same was true at 4 µl/ml of GBE (1195±63 and 1076±23 nmol O/min/mg with and without GBE, respectively).

Further experiments were designed to elucidate the mechanism of GBE-induced uncoupling of oxidative phosphorylation in heart mitochondria. For the assessment of the proton conductance of the mitochondrial inner membrane, we measured spectrophotometrically swelling of non-respiring isolated heart mitochondria in the potassium acetate medium in the presence of valinomycin (Nicholls and Ferguson 1992; Kopustinskiene et al. 2002). Swelling in the absence of valinomycin indicates the permeability of the inner mitochondrial membrane to potassium ions. As it can be seen in Fig. 5, GBE does not affect mitochondrial swelling in the absence of valinomycin. However, in the presence of valinomycin, GBE (1:200, 2 µl and 4 µl/ml) induces mitochondrial swelling in the potassium acetate medium. This suggests that at least some of the compounds present in GBE can act as protonophores.

Proton leak occurs through several pathways (Parker et al. 2008; Harper et al. 2008). A significant part of proton leak is dependent upon the most abundant carrier protein in the mitochondrial inner membrane, ANT, and a lesser part —upon UCP. Therefore, we tested which of these pathways

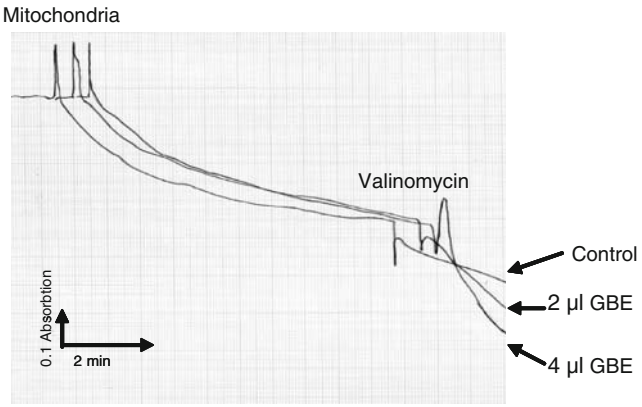

Fig. 5 *Ginkgo biloba* extract (1:200) supported swelling of isolated rat heart mitochondria in potassium acetate medium. $n=2$, with three to four repeats in each

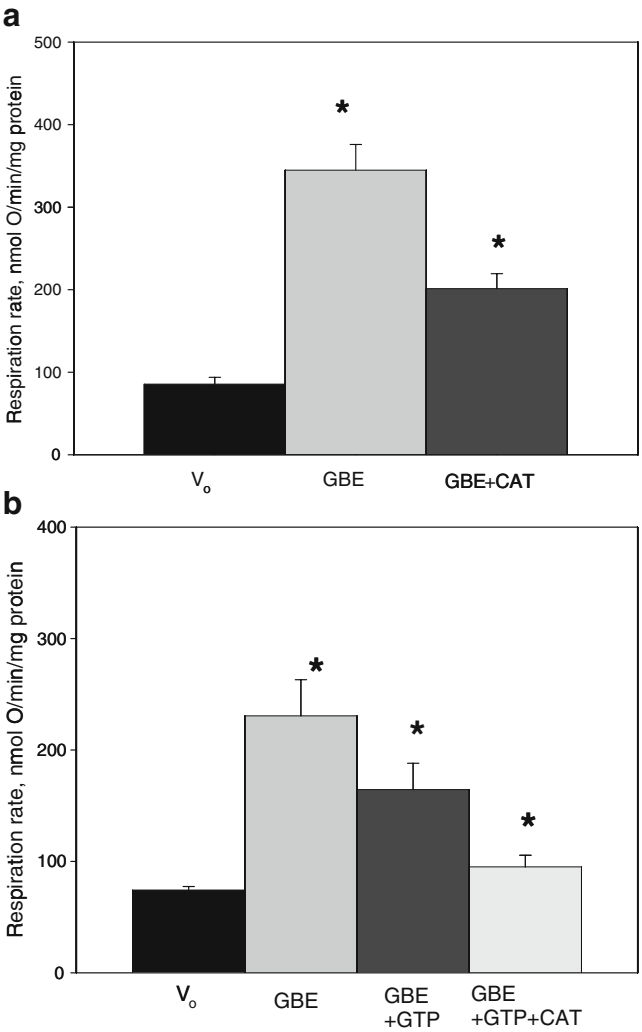

Fig. 6 Effect of CAT and GTP on the respiration of isolated rat heart mitochondria in State 2 stimulated by diluted (1:200) *Ginkgo biloba* extract. Measurements were performed in the presence of 6 mM pyruvate + 6 mM malate as substrates. Standard incubation medium was used. CAT (4 µM) and GTP (30 µM) were added as indicated. *$P<0.05$ vs. Control, $n=6$–7

may be activated by GBE. As can be seen in Fig. 6a, CAT, an ANT inhibitor, significantly decreases the GBE-stimulated respiration of isolated rat heart mitochondria in State 2 (by 41±3%). GTP, an UCP inhibitor, also suppressed (by 29±3.5%) the GBE-increased State 2 respiration though it was less effective than CAT (Fig. 6b). Further addition of CAT decreased the GTP-inhibited respiration by 35%, indicating additive effects of CAT and GTP. This suggests that in heart mitochondria both, ANT and UCP, take part in the mechanism of the GBE-induced increase in proton leak.

We also tested whether GBE affects the H_2O_2 production in isolated rat heart mitochondria. As shown in Fig. 7, GBE (1:200) at concentration of 5 μl/ml clearly suppressed this process: after 10 min incubation GBE caused a 57% reduction in the rate of H_2O_2 generation as compared to the rate in the absence of GBE.

Discussion

In this study, we showed the powerful uncoupling effect of GBE on isolated rat heart mitochondria and permeabilized rat heart fibers respiring on pyruvate + malate: GBE stimulated State 2 respiration of heart mitochondria and significantly decreased mitochondrial membrane potential (Figs. 1, 2). Finding that CAT, an ANT inhibitor, suppresses the GBE-stimulated respiration (Fig. 6a) suggests that a significant part of GBE-induced proton leak in rat heart mitochondria is mediated by ANT. Inhibition of GBE-stimulated respiration by GTP, an UCP inhibitor (Fig. 6b), indicates that UCP is also involved in the mechanism of GBE-induced proton permeability across the mitochondrial inner membrane. Moreover, induction of swelling of non-respiring rat heart mitochondria (inhibited by rotenone) by GBE in potassium acetate medium containing valinomycin (Fig. 5) indicates that at least some of many GBE compounds may act as protonophores. Many of them are polyphenolic substances, mainly flavonoids, and are weak acids of hydrophobic character (Ravanel 1986; van Dijk et al. 2000). Similarly to classical uncouplers of oxidative phosphorylation, they can cause the increase in State 2 respiration rate as it was shown in our previous study (Trumbeckaite et al. 2006). The increase in proton conductance of the mitochondrial inner membrane, in turn, leads to the decrease of mitochondrial membrane potential. However, it should be noted that none of the most abundant flavonoids of GBE (rutin, quercetin, quercitrin) increased State 2 respiration of heart mitochondria to the similar extent as the GBE: pure flavonoids had a maximal 100% effect on the State 2 respiration rate (Trumbeckaite et al. 2006) whereas GBE stimulated the State 2 respiration by about 400% (Fig. 1b). We cannot exclude that much stronger GBE-induced uncoupling of oxidative phosphorylation may be determined by other minor compounds of GBE or by their synergetic action.

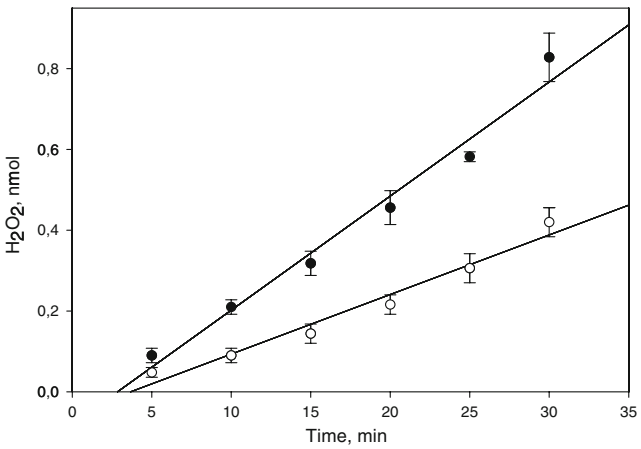

Fig. 7 Effect of diluted (1:200) *Ginkgo biloba* extract on production of H_2O_2 in isolated rat heart mitochondria. Standard incubation medium was used. n=3–4 separate experiments with three repeats in each. In all periods of incubation (exception—5 min), differences were statistically significant (P=0.03). ● without GBE; ○ with GBE (1:200), 5 μl/ml

The differences in the sensitivity of the respiratory function of heart and liver mitochondria to flavonoids have been previously suspected by us investigating the influence of quercetin on the heart mitochondrial respiration (Trumbeckaite et al. 2006). We observed a 2-fold (maximal) stimulation of State 2 respiration of rat heart mitochondria at 3.6 nM quercetin, whereas Dorta et al. (Dorta et al. 2005, 2008) did not observe it in rat liver mitochondria respiring on succinate or glutamate + malate at 25 μM and 50 μM quercetin, i.e. at concentrations more than a thousand times higher. Interestingly, EGb 761 (50–200 μg/ml) had no effect on the respiration of rat liver mitochondria with NAD-linked substrates (ketoglutarate and pyruvate) before anoxia but decreased (by 22%) State 4 respiration after anoxia/reoxygenation (Du et al. 1999). In contrast, we showed earlier (Trumbeckaite et al. 2007) that only a few μl/ml of diluted (1:200) GBE was enough to enhance the heart mitochondrial respiration in State 2 by about 100%. These data imply that rat liver mitochondria are much less sensitive to GBE and its flavonoids than rat heart mitochondria. Thus, it is obvious that the effect of GBE depends on the source of mitochondria. It is also possible that the effect of GBE depends on the type of respiratory substrate. Differences in the action of GBE and its standardized dry extract EGb 761 may be due to differences in composition of these preparations. Though the reasons for differences in the sensitivity of heart and liver mitochondria to GBE are unclear, it is likely that they may be caused by different levels of ANT and UCP in heart and liver mitochondria or by differences in composition of membranes.

Moderate depolarization of isolated mitochondria is known to attenuate generation of ROS in mitochondria (Skulachev 1998; Sack 2006; Halestrap et al. 2007; Miwa and Brand 2003). This may be beneficial in protection against ischemia-reperfusion injury or in other pathological situations associated with oxidative stress. It has been shown *in vivo* that treatment of mice with low doses of the protonophore 2,4-dinitrophenol promotes tissue respiratory rates, decreases ROS levels, tissue DNA and protein oxidation (Caldeira da Silva et al. 2008). We showed that GBE at low concentration reduced $\Delta\Psi$ of rat heart mitochondria by 4–13 mV (Fig. 2). In previous studies by other authors (Skulachev 1998; Miwa and Brand 2003), a similar reduction of $\Delta\Psi$ by classical uncouplers caused the decrease of mitochondrial H_2O_2 production comparable to the effect of GBE observed in our study. We found a 57% reduction of H_2O_2 generation by 5 μl/ml of diluted GBE after 10 min incubation of mitochondria, i.e. after the comparable time for $\Delta\Psi$ measurements (Fig. 7).

Pure flavonoids (quercetin, taxifolin, catechin and galangin) at the concentrations of 1–50 μmol/l are powerful ROS (particularly, superoxide) scavengers (Dorta et al. 2008; Akhlaghi and Bandy 2009). Recently (Masteikova et al. 2007), the radical scavenging activity was shown for the ethanol tincture of *Gingko biloba* leaves in a non-mitochondrial system (12.5 μl/ml, with the 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical cation). However, ROS scavenging possibility seems unlikely or negligible in our study due to very low GBE content and, accordingly, total amount of the main flavonoids—rutin, quercitrin, hyperozide and quercetin—in the medium (17.23 nmol/l). Thus, the GBE-induced reduction of H_2O_2 production in rat heart mitochondria is most likely caused by the reduction of mitochondrial membrane potential.

It is noteworthy that the low concentrations of GBE (few μl/ml of diluted, 1:200) did not affect the State 3 respiration (Trumbeckaite et al. 2007) whereas the higher GBE concentrations (1 μl/ml, undiluted) stimulated respiration, and 4 μl/ml and 6 μl/ml notably depressed it (Fig. 4). GBE-induced inhibition of State 3 respiration was not relieved by CCCP suggesting that GBE may inhibit the activity of respiratory chain complexes or substrate transporters. However, our investigations demonstrated no inhibition of Complex IV activity by 0.2–4 μl/ml of undiluted GBE.

It still remains to be determined whether the direct effects of GBE on mitochondria observed in our *in vitro* studies may occur *in vivo*. This issue is complicated due to various conversions of polyphenols after intake of GBE and uncertainty about the levels of polyphenols accumulating in mitochondria *in vivo* However, since therapeutic doses of GBE (about 5,000 μl, undiluted) are very high compared with the doses effective on isolated mitochondria (a few μl/ml of diluted 1:200 GBE), we believe that this is possible, though the uncoupling effect of GBE *in vivo* should be less than *in vitro* due to very low bioavailability of dietary flavonoids and limited diffusion into (peripheral) tissues (Passamonti et al. 2009) and, therefore, should not be detrimental to the cells. On the other hand, the doses of GBE higher than therapeutic could cause the powerful uncoupling of heart/liver mitochondria and can be deleterious to the cells, therefore their usage should be avoided.

In conclusion, the data obtained in this work clearly demonstrate a dose-dependent uncoupling effect of GBE on isolated rat heart mitochondria, which is caused by protonophoric action of some compounds of GBE and may involve ANT- and UCP-mediated proton conductance of the inner mitochondrial membrane. This GBE-induced proton permeability causes a moderate decrease in mitochondrial membrane potential and attenuation of ROS generation by mitochondria. Rat liver mitochondria appeared to be much less sensitive to GBE than heart mitochondria.

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