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Influence of propolis water solution on heart mitochondrial function

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Abstract

The effect of propolis water solution (PWS) on the respiration of rat heart mitochondria with NAD-linked (pyruvate + malate), FAD-linked (succinate) substrates and fatty acids (palmitoyl-L-carnitine) was investigated in this study. PWS at the lowest concentration of 4 μg mL^{-1} of phenolic compounds (PC) had no effect on mitochondrial respiration with all investigated substrates. PWS at concentrations of 63 and 125 μg mL^{-1} of PC caused a significant decrease of basal (24 and 54%) and maximal (58 and 70%) respiration rates with succinate as substrate. At these PWS concentrations the oxidation of pyruvate + malate and palmitoyl-L-carnitine was diminished to a lower degree: the basal respiration rate decreased by 13–18% and the maximal respiration rate by 15–28%. Succinate oxidation was affected, probably because of the inhibition of succinate dehydrogenase by the 1,2-benzenedicarboxylic acid esters found in PWS. The PWS-caused decrease in the mitochondrial respiration rate with pyruvate + malate and fatty acids could be due to diminished activities of respiratory chain complexes and/or ADP/ATP translocator.

Introduction

In the cardiac muscle cells mitochondria make up 35% of the cell volume and produce 95% of the ATP required for the functioning of the heart. Damage to these organelles may therefore be the key factor for myocardial dysfunction. For this reason, the role of mitochondria in different disorders and the effect of various medical preparations on the mitochondrial processes have been intensively investigated (Brown & Borutaite 2001; Borutaite et al 2003; Neuhof et al 2003). Different bee products have long been used in folk medicine for the treatment of different diseases and only in recent decades have they attracted much scientific interest. Propolis is a complex of natural biologically active substances collected by bees from different plants. Its composition is variable and depends on time, vegetation and area of collection (Velikova et al 2000; Ahn et al 2004; Majiene et al 2004). At present, over 300 natural biologically active components of propolis are known (Marcucci et al 2001).

Because of its antibacterial, antiviral, antifungal, anesthetizing, cytostatic, anti-inflammatory and immune-system-strengthening, hepato-protective effect, propolis and its ethanol solution have long been used for the prevention and treatment of a variety of diseases (Kujumgiev et al 1999; Abd El Hady & Hegazi 2002; Bankova et al 2002; Kartal et al 2002). It has also been shown that propolis has strong antioxidant properties (Ichikawa et al 2002) and therefore it could decrease the damage to different organs caused by free radical production in ischaemia reperfusion. According to literature data, propolis water solution (PWS), compared to an ethanol solution of propolis, suppresses the generation of free radicals (Volpert & Elstner 1996) more effectively. Moreover, water-soluble derivatives of propolis and its polyphenolic compounds significantly reduce the growth and proliferation of tumour cells (Orsolic & Basic 2003). However, the pharmacological effect of PWS as well as the mechanism(s) of its action on cellular energy metabolism have not been sufficiently studied yet. Moreover, there are no data on the effect of propolis on mitochondria. The aim of this study was to investigate the effect of PWS on rat heart mitochondrial respiration.

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Materials and Methods

Quantitative analysis of PWS

Propolis was collected in Lithuania and kept in a dark and dry place. Before extraction propolis was cooled, grated and extracted using distilled water (1:10) at 50°C for 24 h with stirring.

The content of total phenolic compounds in PWS was determined spectrophotometrically (Hitachi 557) by the Folin–Ciocalteu method. Ten millilitres of PWS were added to a 50-mL volumetric flask containing 10 mL of distilled water, 4 mL of Folin–Ciocalteu reagent and 6 mL of 20% sodium carbonate. The volume was made up with distilled water to 50 mL. The colour was developed for 2 h at room temperature and the absorbance was measured at a wavelength of 760 nm. The measurement was compared to a standard curve of prepared gallic acid solution. The results are reported as gallic acid equivalent.

The total phenolics content in our PWS was $1.88\,mg\,mL^{-1}$.

Preparation of rat heart mitochondria

The approval of the Lithuanian Ethic Committee for Laboratory Animal Use was obtained before commencement of the experiments.

Male Wistar rats weighing 250–300 g were used for our study. Rat hearts were excised and rinsed in ice-cold 0.9% KCl solution. Heart mitochondria were isolated in a medium containing 220 mM mannitol, 70 mM sucrose, 5 mM (N-tris[hydroxymethyl]methyl-2-aminoethane) sulfonic acid and 0.5 mM EGTA (pH 7.4, adjusted with Trizma base; 2°C) and $2\,mg\,mL^{-1}$ bovine serum albumin (BSA; fraction V, A4503, Sigma). The homogenate was centrifuged at $750 \times g$ for 5 min, then the supernatant was recentrifuged at $10\,000 \times g$ for 10 min and the pellet was washed once (10 min at $10\,000 \times g$) in the isolation medium without BSA, suspended in it and kept on ice. The mitochondrial protein concentration was determined by the Biuret method using BSA as standard.

Measurement of mitochondrial respiratory rates

Respiration measurements were started immediately after preparation of isolated mitochondria. Oxygen uptake rates of mitochondria were measured at 37°C with a Clark-type electrode in a solution containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM $MgCl_2$, 100 mM 2-[N-morpholino]ethanesulfonic acid, 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), supplemented with $2\,mg\,mL^{-1}$ of BSA. The solubility of oxygen was taken to be $422\,ng\,atoms\,mL^{-1}$. The final mitochondrial protein concentration in all experiments was $0.5\,mg\,mL^{-1}$. Mitochondrial respiration rates were expressed as ng atoms $O\,min^{-1}\,mg\,protein^{-1}$.

Statistical analysis

Data are presented as mean ± s.e.m. Non-parametric methods were applied for making inferences about data. Differences between means in dependent groups were tested using the Wilcoxon matched pairs test. Differences among means in independent groups were tested using the non-parametric Kruskal–Wallis test with Dunn's post-hoc evaluation. $P < 0.05$ was taken as the level of significance. Statistical analysis was performed using the program Statistica 1999, 5.5 StatSoft Inc., USA.

Results

According to our scheme of measurements of rat heart mitochondrial respiration, at the beginning we measured the basal (State 2, in the absence of ADP) respiration rate (Vo) with the respiratory substrates, then after the addition of ADP State 3 (V_{ADP}) respiration was recorded. The respiration was significantly stimulated by ADP, showing that the respiration of mitochondria is tightly coupled with the phosphorylation and synthesis of ATP. Afterwards the State 4 respiration rate (V_{ATR}) was measured after addition of atractyloside. Atractyloside inhibits the ADP/ATP translocator and the access of ADP to mitochondria, and therefore results in a decrease in the State 3 respiration rate. The intactness of the outer mitochondrial membrane (OMM) was easily checked by the subsequent addition of exogenous cytochrome c in the metabolic State 4 (V_{CYT}) (Trumbeckaite et al 2003). The stimulation of respiration by exogenous cytochrome c indicates the loss of cytochrome c from mitochondria due to damage to the OMM, which is inevitable when isolating mitochondria from the cell. Finally, added carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) acts as uncoupler of oxidative phosphorylation and allows the measurement of the maximal respiration rate (V_{CCCP}), which is not related to the synthesis of ATP. The respiration rate with CCCP is higher compared with V_{ADP} (Table 1) because of the stimulating effect of the exogenous cytochrome c, which restores the electron transfer capacity of the respiratory chain in the damaged mitochondria.

The first series of experiments demonstrates the effect of PWS on rat heart mitochondrial respiration with pyruvate + malate, i.e. NAD-specific substrates. The percentage changes in the experimental findings at concentrations of 9–125 $\mu g\,mL^{-1}$ of phenolic compounds (PC) are presented in Figure 1A. Our data show that PWS at concentrations of 9 and 33 $\mu g\,mL^{-1}$ of PC had no effect on the basal and State 3 respiration rates of mitochondria respiration with pyruvate + malate. However, at concentrations of 63 and 125 $\mu g\,mL^{-1}$ of PC, the State 2 respiration rate decreased by 13 and 15%, and the State 3 respiration rate by 26 and 20% ($P < 0.05$). It is noteworthy that a similar effect of PWS (125 $\mu g\,mL^{-1}$ of PC) on State 3 respiration (V_{ADP}) (18% inhibition) was observed in the incubation medium supplemented with cytochrome c ($n = 5$). The respiratory control index (RCI), i.e. the ratio between State 3 and State 2 respiration

Table 1 The effect of different concentrations of PWS on mitochondrial respiration: substrate palmitoyl-L-carnitine

	n	Vo (before adding PWS)	Vo + PWS (after adding PWS)	V_{ADP}	V_{ATR}	V_{CCCP}	V_{ADP}/V_{ATR}
Control	4	155 ± 12	–	513 ± 42	124 ± 14	711 ± 70	4.35 ± 0.59
PC, 9 μg mL^{-1}	3	180 ± 19	167 ± 17	551 ± 64	144 ± 23	783 ± 13	3.99 ± 0.73
PC, 33 μg mL^{-1}	3	175 ± 16	159 ± 15	541 ± 23	144 ± 12	786 ± 31	3.80 ± 0.48
PC, 63 μg mL^{-1}	4	164 ± 25	134 ± 13	426 ± 22	128 ± 18	504 ± 58	3.46 ± 0.52
PC, 125 μg mL^{-1}	4	156 ± 14	133 ± 17	367 ± 20*	123 ± 19	393 ± 63*	3.17 ± 0.58

Measurements were performed in the presence of 8.8 μM palmitoyl-L-carnitine + 0.24 mM malate (Vo); V_{ADP}, respiration rate in the presence of 1 mM ADP; V_{ATR}, respiration rate in the presence of 0.12 mM ATR; V_{CCCP}, respiration rate in the presence of 32 μM cytochrome c and 1.2 μM CCCP; V_{ADP}/V_{ATR}, respiratory control index. Respiratory rates are given as ng atoms O min^{-1} mg protein^{-1}. *$P < 0.05$ vs control; n, number of experiments.

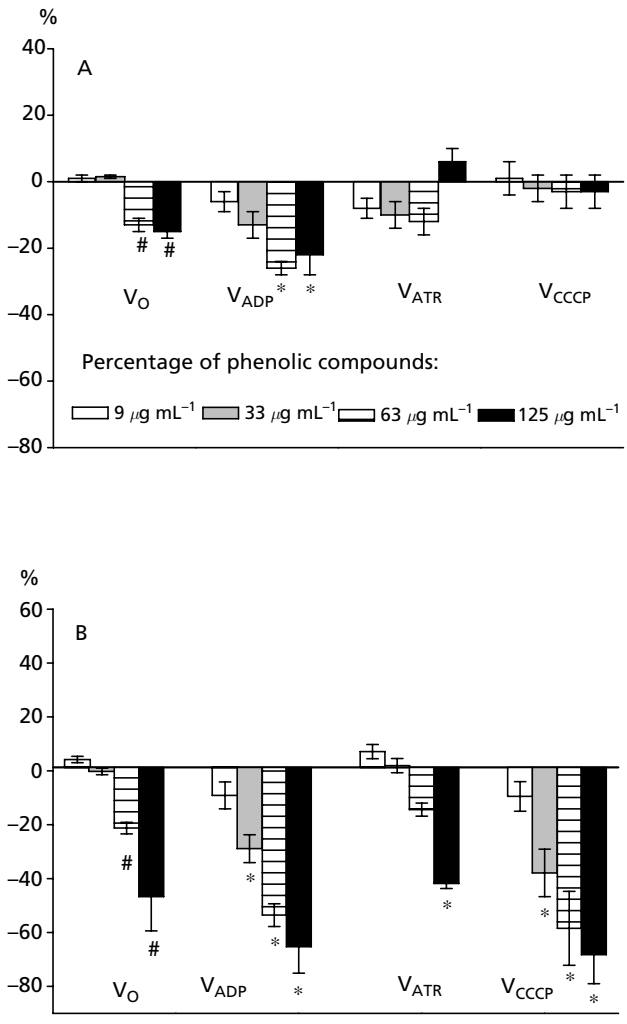

Figure 1 The effect of different concentrations of PWS on mitochondrial respiration with (A) pyruvate + malate and (B) succinate. Vo, (A) pyruvate + malate (6 mM + 6 mM) or (B) succinate + rotenone (10 mM +5 μM); V_{ADP}, 1 mM ADP; V_{ATR}, 0.12 mM ATR; V_{CCCP}, respiration rate in the presence of 32 μM cytochrome c and 1.2 μM CCCP; number of experiments = 5; $P < 0.05$ vs Vo; *$P < 0.05$ vs control.

rates, was also diminished (control, 4.27 ± 1.1; 125 μg mL^{-1} of PC, 3.23 ± 0.6). However, this decrease was not statistically significant since the State 3 respiration rate as well as the State 2 respiration rate decreased to a similar extent. The other parameters, V_{ATR}, V_{CYT} (not shown) and V_{CCCP}, were not affected significantly by PWS.

Other experiments aimed to check the action of PWS on FAD-specific substrate–succinate oxidation. As seen in Figure 1B, the State 2 respiration rate (Vo) did not differ from that of the control group at concentrations of 9 and 33 μg mL^{-1} of PC, while the State 3 respiration rate decreased by 12 and 31%, respectively, compared to the control. These changes were not statistically significant. In the presence of higher concentrations of PWS (63 and 125 μg mL^{-1} of PC), Vo decreased by 24 and 54%, V_{ADP} by 58 and 70% and V_{ATR} (a parameter analogous to Vo) by 26 and 54%, $P < 0.05$. Because of a more pronounced decrease in V_{ADP} than Vo, the RCI decreased by 38%, i.e. from 1.60 ± 0.03 (control) to 1.01 ± 0.2 and 1.0 ± 0.1 (with 63 and 125 μg mL^{-1} of PC, respectively; $P < 0.05$). Addition of exogenous cytochrome c in State 4 equally stimulated respiration (by *c.* 40–60%) in all groups as well as in the control. This shows that PWS did not affect the integrity of the OMM. Interestingly, and in contrast to pyruvate + malate, in the presence of PWS, V_{CCCP} decreased similarly to V_{ADP} in the case of succinate oxidation.

Our studies of N, N, N,′N′-tetramethyl-*p*-phenylenediamine and ascorbate oxidation showed that PWS had no effect on the activity of complex IV, also called cytochrome oxidase (control, 2913 ± 136 ng atoms O min^{-1} mg protein^{-1}; in the presence of 125 μg/mL of PC, 2953 ± 123 ng atoms O min^{-1} mg protein^{-1}, n = 3).

Since fatty acids are the main respiratory substrates of the heart, further experiments were performed to study the effect of PWS on the oxidation of palmitoyl-L-carnitine, the activated derivative of palmitate. The results presented in Table 1 show that the effect of PWS on the basal respiration rate with palmitoyl-L-carnitine is not statistically significant. However, its decrease (7–18%) after addition of PWS, even at low concentrations (9 and 33 μg mL^{-1} of PC), was observed in all measurements,

which is in contrast to the oxidation of pyruvate and succinate. The decrease in the maximal mitochondrial respiration rate (V_{ADP} and V_{CCCP}) by 28 and 44%, respectively, was achieved only at the maximal concentration of PWS (125 $\mu g\,mL^{-1}$) used in our experiments.

It should be mentioned that the lowest used concentration of PWS (4 $\mu g\,mL^{-1}$ of PC) had no effect on the mitochondrial respiration with all investigated substrates (data not shown). Furthermore, we obtained similar results with two other samples of propolis, collected in different parts of Lithuania (data not shown).

The doses of various propolis preparations used by different authors are different and differently expressed, therefore it is difficult to compare the data. Nevertheless, it seems likely that the concentrations of PWS used in our study (4–125 $\mu g\,mL^{-1}$ PC) are comparable to the therapeutic concentrations used by different authors (Nagai et al 2003; Orsolic & Basic 2003).

The results described above demonstrate that succinate oxidation was mostly affected by PWS, as compared to other substrates. Our data from the GC-MS analysis of the chemical composition of PWS (Trumbeckaite et al 2004) show that PWS contains esters of 1,2-benzenedicarboxylic acid. According to findings presented in literature (Melnick & Schiller 1982, 1985), esters of 1,2-benzenedicarboxylic acid (phthalic acid) inhibit the State 3 respiration of rat liver mitochondria, oxidizing succinate, succinate–cytochrome c reductase (complex II + III) and succinate dehydrogenase. Esters of 1,2-benzenedicarboxylic acid are ascribed to non-competitive inhibitors of succinate dehydrogenase (Melnick & Schiller 1985). Thus we assume that the propolis-induced decrease in the State 3 respiration rate of rat heart mitochondria with succinate could be due to the inhibition of succinate dehydrogenase and/or succinate–cytochrome c reductase conditioned by esters of 1,2-benzenedicarboxylic acid.

The effect of PWS on the oxidation of palmitoyl-L-carnitine and pyruvate + malate was lower than that on the oxidation of succinate. It is known that palmitoyl-L-carnitine oxidation involves the citric acid cycle and succinate dehydrogenase. The possibility exists therefore that PWS-induced suppression of fatty acid oxidation in mitochondria might be, at least to some extent, related to the inhibition of succinate dehydrogenase and/or other respiratory chain complexes. On the other hand, since CCCP released the PWS-induced inhibition of ADP-stimulated (State 3) respiration of mitochondria only with pyruvate + malate, one may conclude that the inhibition of pyruvate + malate oxidation may be due to inhibition of ADP/ATP translocator and/or ATPase.

Some recent publications have indicated the antitumour and antimetastatic activity of propolis (El khawaga et al 2003; Russo et al 2004; Kuo et al 2005). It was proposed that the latter could be partially related to propolis apoptotic activity and non-specific stimulation of macrophages (Orsolic & Basic 2003). Since mitochondria are specialized for energy production and also participate in cell cycle regulation and apoptosis (Borutaite & Brown 2003), the propolis-induced impairment of mitochondrial respiration and energy production found in our study could be regarded as one of the possible mechanisms for propolis cytostatic activity.

The antimicrobial activity of propolis has been widely reported; however, the mechanisms of its action are not well known. Interestingly, the mechanism of energy transduction is similar in mitochondria and in bacteria; therefore one may assume that propolis might inhibit bacterial respiration and energy production. Our preliminary data were in agreement with this assumption. Since the bacteria use energy concentrated in the bond of ATP for their growth and reproduction (Tortora et al 2004), the inhibition of ATP synthesis in bacteria may be the reason for the antimicrobial action of propolis.

The results of our study showed that the inhibitory effects of PWS on mitochondrial respiration not only depend on the oxidized substrate but also on the concentration of PWS. However, further studies are needed for the clarification of the mechanisms of this phenomenon.

Conclusion

PWS at the lowest concentration used in our experiments of 4 $\mu g\,mL^{-1}$ of PC had no effect on rat heart mitochondrial respiration with all investigated substrates. Oxidation of pyruvate + malate and palmitoyl-L-carnitine at concentrations of 63 and 125 $\mu g\,mL^{-1}$ of PC was diminished: the basal rate by 13–18% and the maximal ADP-stimulated rate by 15–28%. The oxidation of succinate was mostly affected by PWS. The basal rate decreased by 24 and 54% and the maximal ADP-stimulated respiration rate by 58 and 70%, respectively. The reasons for the inhibition of mitochondrial respiration seem to be different with different respiratory substrates. All concentrations of PWS used in our experiments did not change the integrity of the OMM.

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