ISBN 978-85-61091-05-7

VI EPCC
Encontro Internacional de Produção Científica Cesumar
27 a 30 de outubro de 2009

THE EFFECTS OF CARBENOXOLONE ON ENERGY METABOLISM OF RAT LIVER MITOCHONDRIA

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ABSTRACT: Carbenoxolone is a derivative of glycyrrhetinic acid, the active principle of licorice (*Glycyrrhiza glabra*), a medicinal root. The pharmacological properties attributed to carbenoxolone are related to its inhibitory actions on the 11β-hydroxysteroid dehydrogenase and gap junction channels. Recent studies have shown that carbenoxolone also induces swelling and membrane potential collapse in mitochondria. These effects were related to hydrogen peroxide generation and mitochondrial permeability transition (MPT) induction, indicating possible toxicological actions of carbenoxolone at the mitochondrial level, which could trigger the apoptotic pathway. The data of these previous reports are pointing, thus, in the direction of a possible action of carbenoxolone on the bioenergetic functions of mitochondria, which could in turn cause toxic metabolic changes in the liver. For this reason, the present work was planned to investigate the action of carbenoxolone on respiratory and ATPase activity of isolated rat liver mitochondria. Male Wistar rats, weighing 180 to 220 g, fed with a standard laboratory diet were utilized. Mitochondrial respiration was measured polarographically. The mitochondrial ATPase activity was measured in intact (coupled and uncoupled) and in freeze-thawing disrupted mitochondria**.** In isolated mitochondria, carbenoxolone increased state IV respiration and respiration dependent solely on succinate and β-hydroxybutyrate oxidation. However, it decreased state III respiration and diminished the respiratory control ratio. Carbenoxolone stimulated the ATPase activity of intact mitochondria and inhibited the ATPase activity of uncoupled mitochondria. The ATPase activity of freeze-thawing disrupted mitochondria was not altered. Carbenoxolone impairs energy metabolism probably acting as an uncoupler of oxidative phosphorylation.

KEY WORDS: Carbenoxolone; Metabolism; Mitochondria.

1 INTRODUCTION

Carbenoxolone is a derivative of glycyrrhetinic acid, the active principle of licorice (*Glycyrrhiza glabra*), a medicinal root. The pharmacological properties attributed to carbenoxolone are related to its inhibitory actions on the 11β-hydroxysteroid dehydrogenase and gap junction channels (DAVIDSON; BAUMGARTEN, 1988). It was observed that carbenoxolone, by blocking the gap junctional intercellular communications (GJIC) reduces the efficiency of tumor cell diapedesis, a process involved in the migration of these malignant cells (metastasis). In addition to blocking the gap junctional intercellular communications, glycyrrhetinic acid and its derivatives exhibit anti-inflammatory

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(GOLDBERG et al., 1996), antiulcerous and antiviral activities. Recent studies have shown that carbenoxolone also induces swelling and membrane potential collapse in mitochondria. These effects were related to hydrogen peroxide generation and mitochondrial permeability transition (MPT) induction, indicating possible toxicological actions of carbenoxolone at the mitochondrial level, which could trigger the apoptotic pathway. Since impairment of the bioenergetic capacity of mitochondria could cause metabolic changes in the liver, the present work was undertaken to investigate the action of carbenoxolone on isolated rat liver mitochondria.

2 MATERIALS AND METHODS

All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá.

Fed rats, weighing between 180 and 220 g, were decapitated and their livers removed immediately and cut into small pieces. These fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 1.0 mM Tris-HCl (pH 7.4), 1 mM ethlylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 50 mg% fatty acid-free bovine serum albumin. Homogenization was carried out in the same medium by means of a van Potter-Elvehjem homogenizer. After homogenization the mitochondria were isolated by differential centrifugation and suspended in the same medium, which was kept at 0-4^{o}C.

Oxygen uptake by isolated mitochondria was measured polarographically using a teflon shielded platinum electrode. Mitochondria (0.85±0.35 mg protein/ml) were incubated in the closed oxygraph chamber in a medium (2.0 ml) containing 0.25 M mannitol, 5 mM sodium phosphate, 10 mM KCl, 0.2 mM EDTA, 25 mg% fatty acid-free bovine serum albumin, 10 mM Tris-HCl (pH 7.4) and two different substrates in addition to various carbenoxolone concentrations in the range between 20 and 200 μM. The substrates were succinate (10 mM) and β-hydroxybutyrate (10 mM). ADP, for a final concentration of 0.125 mM, was added at appropriate times. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as nmol per minute per mg protein. The respiratory control ratio (RC) and the ADP/O ratio were calculated. Protein content of the mitochondrial suspensions was measured by means of the method described by Lowry et al (1951), using the Folin-phenol reagent and bovine-serum albumin as a standard.

The mitochondrial ATPase activity was measured in intact (coupled and uncoupled) and in freeze-thawing disrupted mitochondria. When intact mitochondria were used as enzyme source, the reaction medium contained: 0.2 M sucrose, 10 mM TRIS-HCl (pH 7.4), 50 mM KCl, 0.2 mM EGTA and, when required, 0.2 mM 2,4-dinitrophenol (uncoupled mitochondria). When disrupted mitochondria were incubated, the medium contained 20 mM TRIS-HCl (pH 7.4). The reaction was started by the addition of 5 mM ATP and stopped, after 20 min of incubation at 37°C, by the addition of ice-cold 5% trichloroacetic acid. Phosphate was measured as described by Fiske and Subbarow (1925).

The statistical significance of the differences between parameters obtained in the experiments was evaluated by means of Newman-Keuls test after submitting the data to variance analysis according to context. The results are mentioned in the text as the p values; $p < 0.05$ was the criterion of significance.

3 RESULTS AND DISCUSSION

If the action of carbenoxolone on hepatic oxygen uptake is of mitochondrial origin it should be possible, in principle at least, to reproduce these effects in isolated mitochondria. For this purpose the influence of carbenoxolone on the respiration of isolated rat liver mitochondria was measured using two different substrates as electron

donnors. As revealed by Figure 1 (A and B) these substrates were succinate (FAD-dependent) and β-hydroxybutyrate (NAD^+-dependent). The mitochondrial respiration driven by the oxidation of these substrates was measured in isolated rat liver mitochondria incubated in the absence of exogenous ADP (substrate respiration), presence of exogenous ADP (state III respiration) and after the exhaustion of the exogenously added ADP (state IV respiration) in the absence and presence of several carbenoxolone concentrations.

Irrespective of the substrate that was used in the assays, carbenoxolone affected oxygen uptake of isolated mitochondria only at high concentrations. Figure 1A and 1B reveals that before ADP addition (substrate respiration) and after exhaustion of ADP (state IV respiration) carbenoxolone increased oxygen uptake in a dose-dependent manner with both substrates (succinate and β-hydroxybutyrate). In the presence of ADP (state III respiration) carbenoxolone caused inhibition of oxygen uptake at the concentrations of 160 and 200 μM. Furthermore, the respiratory control was almost abolished, as can be judged from the state IV respiration rates and from the respiratory control ratios (state IV/state III) in Table 1. The ADP/O ratios were also evaluated and listed in Table 1. They were not affected by carbenoxolone when succinate was the substrate. Significant effects were seen with β-hydroxybutyrate only at the highest carbenoxolone concentrations (160 and 200 μM).

The effects of carbenoxolone on the ATPase activity were measured in intact mitochondria either in the absence (coupled mitochondria) or in the presence of 2,4-dinitrophenol (uncoupled mitochondria) and in freeze-thawing disrupted mitochondria, as shown in Figure 2. The actions of carbenoxolone were different in each preparation. The ATPase activity of coupled mitochondria was increased over the whole concentration range of carbenoxolone. Numerical interpolation reveals that 100% stimulation can be expected at a carbenoxolone concentration of 21.8 μM. The ATPase activity of uncoupled mitochondria, on the other hand, was inhibited. This inhibition was significant within the concentration range of 160 to 200 μM. When disrupted mitochondria were used as the enzyme source, the ATPase activity was not significantly affected by carbenoxolone.

Table 1. Action of Carbenoxolone on Mitochondrial Respiration Driven by Succinate or β-Hydroxybutyrate in the Presence and Absence of Exogenously Added ADP.

	β-Hydroxybutyrate (n = 8)		*Succinate (n = 7)*	
Carbenoxolone (μM)	*ADP/O*	*Respiratory control ratio*	*ADP/O*	*Respiratory control ratio*
0	2.85 ± 0.27	6.58 ± 0.55	1.66 ± 0.13	5.97 ± 0.62
20	2.80 ± 0.20	4.07 ± 0.55[a]	1.90 ± 0.16	5.55 ± 0.53
50	2.55 ± 0.28	3.42 ± 0.35[a]	1.81 ± 0.21	4.65 ± 0.49
100	2.49 ± 0.39	2.94 ± 0.35[a]	1.76 ± 0.22	3.49 ± 0.37[a]
160	1.57 ± 0.54[a]	1.21 ± 0.11[a]	1.66 ± 0.25	2.39 ± 0.58[a]
200	–[b]	1.02 ± 0.02[a]	1.51 ± 0.31	1.53 ± 0.44[a]

Mitochondria were isolated and assayed as described in the Materials and Methods section. Incubations were done in the presence of substrate (10 mM) as indicated. Values are means ±standard errors. a Statistically significant relative to the controls (variance analysis with post hoc Newman–Keuls testing; $p < 0.05$). bVery low value, no accurate determination possible.

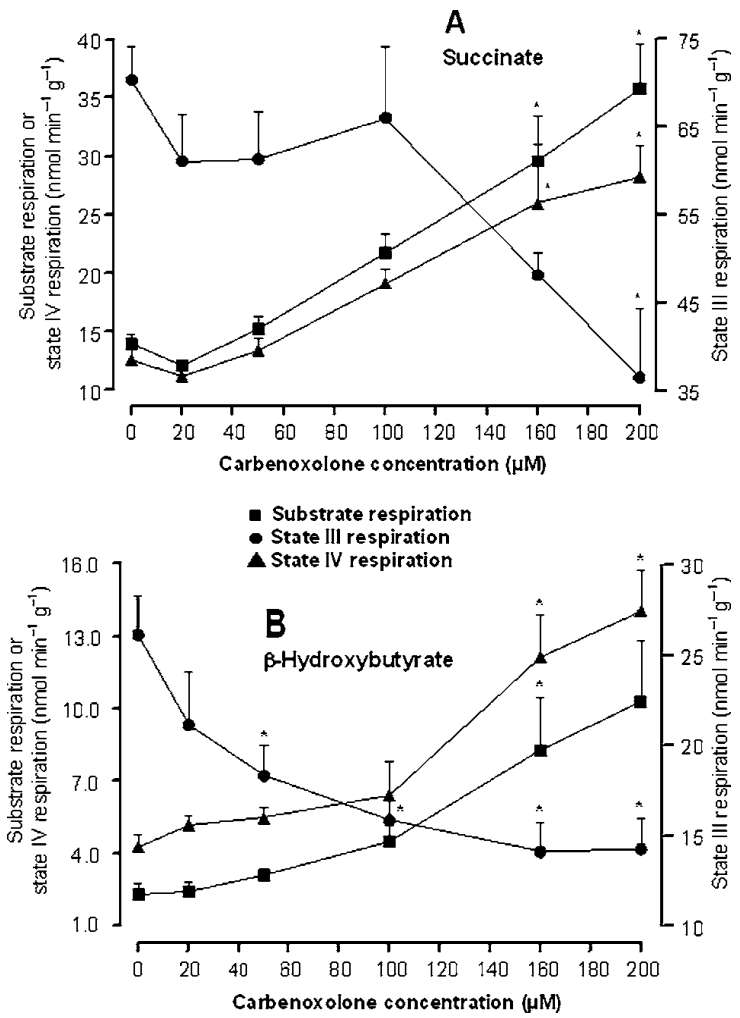

Figure 1. Effects of carbenoxolone on the respiratory activity of isolated rat liver mitochondria. Mitochondria (0.25–2.5mg/mL)were added to the reaction medium in the closed vessel of the oxygraph. The reaction was initiated by the addition of succinate (A) or β-hydroxybutyrate (B), and the oxygen consumption was followed polarographically for 5 min. After this time 0.25–0.5 nmol of ADP was added. Rates of oxygen consumption were computed from the slopes of the polarographic records. Each datum point is the mean ± SEM of four independent experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Newman–Keuls test ($p < 0.05$).

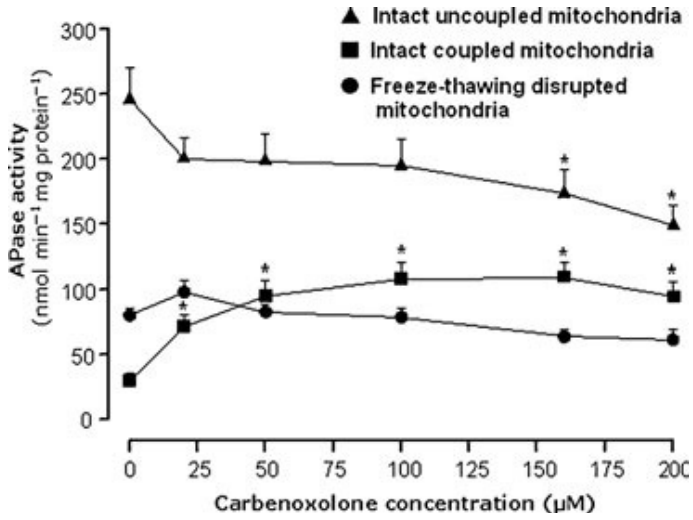

Figure 2. Effects of carbenoxolone on the ATPase activity of coupled, uncoupled, and disrupted mitochondria. The mitochondria were incubated at 37◦C in reaction medium as described in the Materials and Methods section. Each assay point represents the mean of eight (coupled mitochondria), seven (0.2 mM 2,4-dinitrophenol uncoupled mitochondria), and eight (freeze-thawed disrupted mitochondria) independent experiments and each datum point is the mean ± SEM. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Newman–Keuls test ($p < 0.05$).

4 CONCLUSION

The data obtained with isolated mitochondria corroborate the conclusion that carbenoxolone impairs energy metabolism probably acting as an uncoupler of oxidative phosphorylation. The uncoupling action is indicated by its effects on mitochondrial respiration and ATPase activity, namely stimulation of state IV respiration and substrate respiration, decrease of the respiratory control ratio and increase of ATP hydrolysis in intact and coupled mitochondria.The effects of carbenoxolone on the ATPase activity of different mitochondrial preparations, however, suggests that, in addition to the uncoupling action, the drug could also be a weak inhibitor of the ATP/ADP exchange system.

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