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The Action of Quercetin on the Mitochondrial NADH to NAD^+ Ratio in the Isolated Perfused Rat Liver

Original Paper

Abstract

It has been suggested that active forms of quercetin (*o*-semiquinones) are able to oxidize NADH in mammalian cells. The purpose of this study was to investigate this proposition by measuring the β-hydroxybutyrate to acetoacetate ratio as an indicator of the mitochondrial NADH/NAD^+ redox ratio in the isolated perfused rat liver. The NADH to NAD^+ ratio was reduced by quercetin; half-maximal reduction occurred at a concentration of 32.6 μM. Additionally, quercetin (25 to 300 μM) stimulated the Krebs cycle ($^{14}CO_2$ production) and inhibited oxygen uptake (50 to 300 μM). Low quercetin concentrations (25 μM) stimulated oxygen uptake. The results of the present work confirm the hypothesis that quercetin is able to participate in the oxidation of NADH in mammalian cells, shifting the cellular conditions to a more oxidized state (prooxidant activity). Stimulation of the Krebs cycle was probably caused by the increased NAD^+ availability whereas the decreased NADH availability and the inhibition of mitochondrial energy transduction could be the main causes for oxygen uptake inhibition.

Key words
Quercetin · liver · prooxidant activity · NADH oxidation

Introduction

Quercetin is a flavonoid that contains a catechol ring in its structure [1], [2]. This compound exerts a variety of short- and long-term effects in tumor and mammalian cells. Inhibition of glycolysis in tumor and rat testis cells is one of these effects [3], [4]. This action has been related to the highly effective inhibition of the Na^+-K^+-adenosine triphosphatase, a phenomenon that produces low cellular concentrations of ADP and inorganic phosphate. Meanwhile, it is known that quercetin inhibits the proliferation of a variety of malignant cells in culture [5]. The mechanisms of this action are not fully understood, but they include inhibition of tyrosine kinase [6], inhibition of heat-shock proteins [7], inhibition of the expression of ras-proteins [8] and several additional effects [5].

In the liver from fed rats, quercetin stimulates glycogenolysis, oxygen consumption and glycolysis at concentrations up to 300 μM [9]. Complex effects were observed at higher concentrations. Besides this, quercetin inhibits gluconeogenesis and oxygen uptake in livers from fasted rats [10]. Decreased efficiency of mitochondrial energy transduction and inhibition of several enzymes besides the Na^+-K^+-adenosine triphosphatase seem to be the main primary causes of the metabolic effects of quercetin. In the liver, it has also been observed that quercetin reduces the lactate to pyruvate ratio, which is an indicator of the cytosolic NADH to NAD^+ ratio [9], [10]. The origin of this phenomenon could be NADH oxidation in consequence of the interaction of quercetin with cellular enzymes. In fact, it has been shown that peroxidases can generate *o*-semiquinones from quercetin and other catechol ring-containing polyphenolics with stoichiometric hydrogen peroxide reduction [1], [11]. These *o*-semiquinones can cause NADH oxidation, without oxygen consumption [1], [2],

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[12]. It is known that the liver possesses enzymatic systems capable of producing free radicals from phenolic or polyphenolic compounds [13], [14], [15], [16].

If quercetin changes the cytosolic NADH to NAD^+ ratio because it causes the oxidation of NADH, then it should affect the mitochondrial NADH to NAD^+ ratio in the same way. This was the main question in the present work in which the action of quercetin on the mitochondrial NADH to NAD^+ ratio was investigated. The β-hydroxybutyrate to acetoacetate ratio in the liver of fasted rats was utilized as an indicator for the mitochondrial NADH/NAD^+ ratio [17], [18]. This is possible because the enzyme β-hydroxybutyrate dehydrogenase is present solely in the mitochondria and also because it operates under near-equilibrium conditions [17]. Direct measurements of the mitochondrial NADH/NAD^+ ratio are very difficult to perform. This highly dynamic parameter is strictly dependent on the cellular conditions, which are changed immediately as a result of organelle isolation. The action of quercetin on parameters related to energy metabolism was also investigated because these parameters and the NADH-NAD^+ redox potential present important interrelationships. A more detailed characterization of the potential prooxidant effects of quercetin is important because this substance has generally been considered to be an effective antioxidant, a property that is often emphasized when its anticancer properties are analyzed [5], [19].

Materials and Methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá [20]. Enzymes and coenzymes used in the assay procedures and quercetin were purchased from Sigma Chemical Co. (St. Louis, USA). The minimum purity of quercetin was 98%, as determined by high-performance liquid chromatography. Labeled octanoate ([1 – ^{14}C]octanoate) was purchased from Amersham International (Buckinghamshire, England). All other chemicals were of the highest available grade.

Liver perfusion

Male Wistar rats (weighing 200 – 280 g) fed with a standard laboratory diet (Nuvilab®) were used in all experiments. Food was withdrawn 24 hours prior to the perfusion experiments. For the surgical procedure, rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). Hemoglobin-free, non-recirculating perfusion was performed [21]. The flow was adjusted to between 30 and 35 mL min^{-1}, depending on the liver weight. The perfusion fluid was Krebs/Henseleit bicarbonate buffer, pH 7.4 [21], saturated with a mixture of oxygen and carbon dioxide (95 : 5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 °C. Livers from 24-hours fasted rats have very low glycogen levels, and in isolated perfusion they survive at the expense of endogenous fatty acids [22]. Substrate-free perfusion fluid was used, and trace amounts of [1 – ^{14}C]octanoate were infused from the beginning of the perfusion experiments in order to label the Krebs cycle intermediates via acetyl-CoA [23]. The production of $^{14}CO_2$ under these conditions is a measure of the activity of the Krebs cycle. Quercetin was dissolved into the perfusion fluid.

Analytical methods

The oxygen concentration in the effluent perfusate was monitored polarographically, employing a teflon-shielded platinum electrode [24]. Acetoacetate and β-hydroxybutyrate in the outflowing perfusate were measured enzymatically [25], [26]. Appropriate control experiments revealed that quercetin did not affect the soluble β-hydroxybutyrate dehydrogenase used in the assay of β-hydroxybutyrate and acetoacetate [26]. The carbon dioxide production from [1 – ^{14}C]octanoate was measured by trapping $^{14}CO_2$ in phenylethylamine [27]. Radioactivity was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/ethanol (2/1) containing 5 g/L 2,5-diphenyloxazole and 0.15 g/L 2,2-*p*-phenylenebis(5-phenyloxazole).

Experiments with isolated rat liver mitochondria

Rats weighing between 200 and 280 g were decapitated, their livers removed and homogenized (van Potter–Elvehjem homogenizer) in a medium containing 0.2 M mannitol, 75 mM sucrose, 1.0 mM Tris-HCl (pH 7.4), 1 mM ethylene glycol bis(β-aminoethyl ether)tetraacetic acid (EGTA) and 50 mg% fatty acid-free bovine serum albumin. The mitochondria were isolated by differential centrifugation [28], [29] and suspended in the same medium at 0 – 4 °C. Protein content was measured by means of the Folin phenol reagent [30], using bovine serum albumin as a standard.

Oxygen uptake by isolated mitochondria was measured polarographically using a teflon-shielded platinum electrode [24], [28]. 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 disodium phosphate, 10 mM KCl, 0.2 mM EDTA, 25 mg% fatty acid-free bovine serum albumin, 10 mM Tris-HCl (pH 7.4), substrates and quercetin (0, 150 and 300 μM). The substrates were succinate (10 mM), citrate (10 mM), β-hydroxybutyrate (10 mM) and α-ketoglutarate (10 mM). Quercetin was added as a dimethylformamide solution. Appropriate control experiments were performed in order to exclude solvent effects. ADP, at a final concentration of 125 μM, was added at appropriate times in order to evaluate the action of quercetin on oxidative phosphorylation. Rates of oxygen uptake were computed from the slopes of the recorder tracings. The ADP/O ratios and the respiratory control ratios (RC) were computed from the recorder tracings [31].

Data analysis

Data were shown as means ± standard errors of the mean (SEM). Statistical significance of the differences between parameters was evaluated by means of Student's t test or Student-Newman-Keuls test after variance analysis, according to context; $p \leq 0.05$ was adopted as a criterion of significance.

Results

The results of the experiments in which the actions of quercetin on ketogenesis (β-hydroxybutyrate and acetoacetate productions), oxygen uptake and $^{14}CO_2$ production were measured are shown in Figs. **1** and **2**. Fig. **1** illustrates the experimental protocol and shows the time courses of the changes caused by 300 μM quercetin. Zero time corresponds to the instant at which sam-

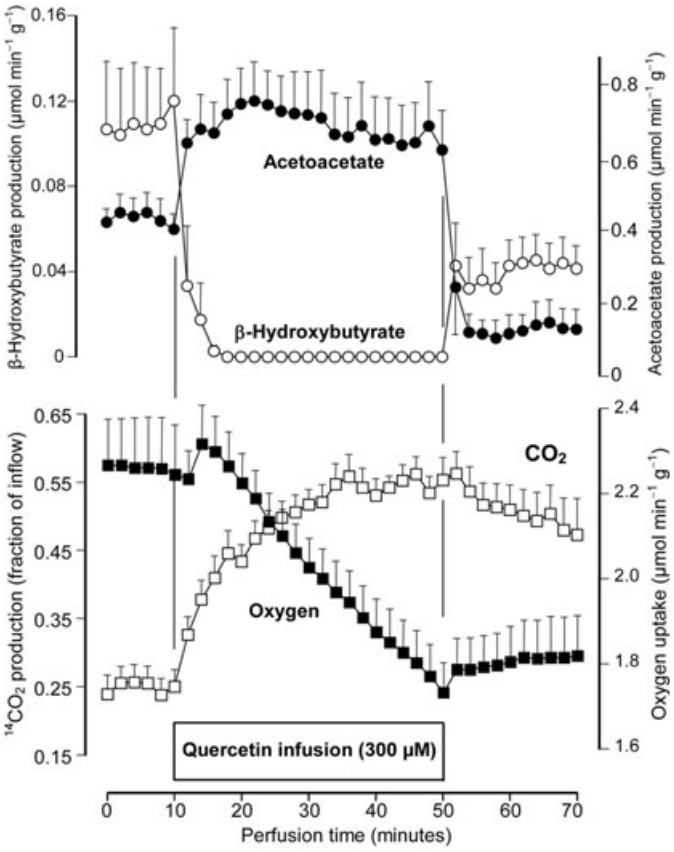

Fig. **1** Time course of the changes caused by 300 μM quercetin on ketogenesis, oxygen uptake and $^{14}CO_2$ production in the perfused rat liver. The data are the means of 5 liver perfusion experiments. Vertical bars are mean standard errors.

pling of the outflowing perfusate was initiated. Ten minutes after initiation of perfusate sampling, the quercetin infusion was started and continued for the next 40 minutes. All metabolic fluxes responded to quercetin. The production of β-hydroxybutyrate was rapidly decreased to non-detectable levels by 300 μM quercetin (Fig. **1**). Acetoacetate production was stimulated to a relatively small extent. Oxygen uptake was stimulated only slightly and transiently just after starting the infusion. Following this increase, it decreased continuously during the whole infusion period and, apparently, the process was still in progress when the quercetin infusion was interrupted. The production of $^{14}CO_2$ was increased. When the infusion of quercetin was interrupted, only the production of acetoacetate and β-hydroxybutyrate showed an immediate reaction with a partial recovery. Oxygen uptake and $^{14}CO_2$ production showed little, if any, tendency toward recovery during the subsequent 20 minutes.

The experiments with 300 μM quercetin illustrated in Fig. **1** were repeated with 25, 50 and 100 μM quercetin in order to evaluate the concentration dependence of the effects. The mean results are summarized in Fig. **2**. The metabolic rates that were measured at 40 minutes after starting quercetin infusion were plotted against the perfusate quercetin infusion. The values at zero quercetin concentration correspond to the mean basal rates of all experiments. Fig. **2** reveals that oxygen uptake was slightly stimulated by 25 μM quercetin and inhibited at higher concentrations. The production of $^{14}CO_2$, on the other hand, was stimulated over the whole concentration range. Numerical interpolation predicts that 100% stimulation can be expected at a concentration of 147.5 μM (Stineman's interpolation). The total production of ketone bodies (β-hydroxybutyrate + acetoacetate) was decreased at low quercetin concentrations (up to 50 μM), but this inhibition was no longer present at the highest concentration (300 μM). The β-hydroxybutyrate to acetoacetate ratio was reduced with a clear concentration dependence. Half-maximal reduction can be expected at a concentration of 32.6 μM, as revealed by numerical interpolation (Stineman's interpolation).

If the action of quercetin on hepatic oxygen uptake is of mitochondrial origin, it should be possible to reproduce these effects in isolated mitochondria. With this purpose in mind the influence of quercetin on respiration of isolated rat liver mitochondria was measured using four different substrates as electron donors. As shown in Table **1**, these substrates were citrate, β-hydroxybutyrate, α-ketoglutarate and succinate. Irrespective of the substrate that was used in the assays, quercetin did not affect oxygen uptake of isolated mitochondria before ADP addition (substrate respiration in Table **1**). In the presence of ADP (state

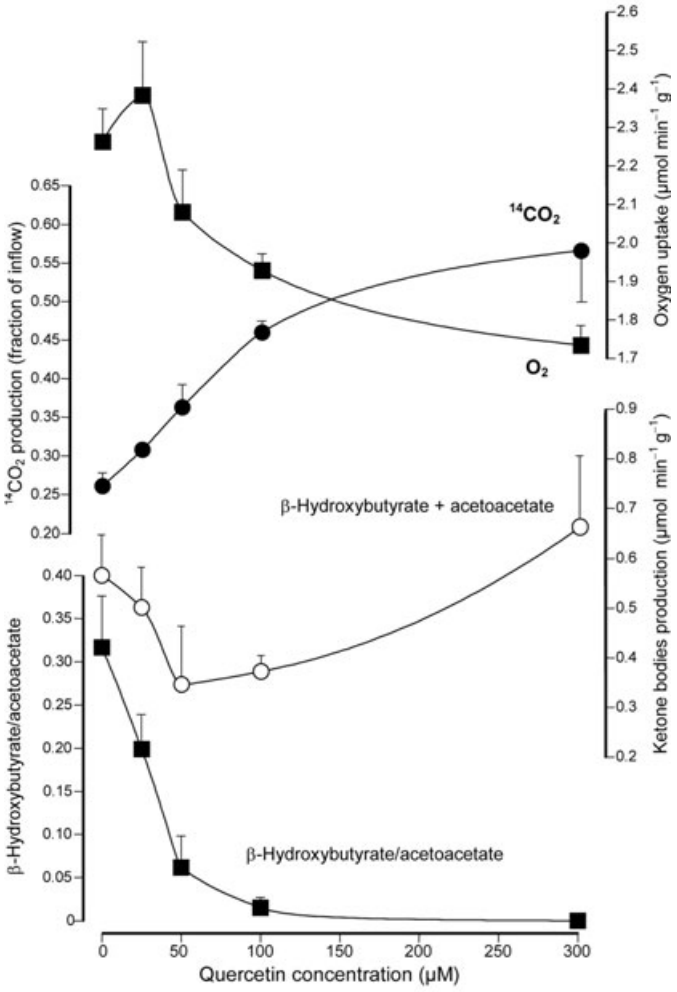

Fig. **2** Concentration dependencies of the effects of quercetin in the isolated perfused liver from fasted rats. Data were obtained from experiments similar to that one illustrated by Fig. **1**. Values in the absence of quercetin (control values) are the mean values before the onset of quercetin infusion. Values in the presence of quercetin were computed at the end of the infusion. Data are the means ± mean standard errors.

Table **1** The action of quercetin on mitochondrial respiration driven by various substrates in the presence and absence of exogenously added ADP

Substrate	*Quercetin concentration (μM)*	*Substrate respiration*	*State III respiration*	*State IV respiration*	*Respiratory control ratio*	*ADP/O Ratio*
		(μmol O_2 min^{-1} mg $protein^{-1}$)				
Citrate (n = 5)	0	3.67 ± 0.22	7.33 ± 0.54	3.66 ± 0.45	2.14 ± 0.05	2.46 ± 0.05
	150	3.34 ± 0.19	4.05 ± 0.49*	4.05 ± 0.49	1.0*	–
β-Hydroxybutyrate (n = 3)	0	3.08 ± 0.15	11.02 ± 0.89	4.29 ± 0.24	2.63 ± 0.06	2.86 ± 0.01
	150	2.65 ± 0.10	6.42 ± 0.44*	6.42 ± 0.44*	1.0*	–
	300	2.97 ± 0.27	4.08 ± 0.11*	4.08 ± 0.11*	1.0*	–
α-Ketoglutarate (n = 5)	0	5.27 ± 0.46	15.76 ± 1.11	6.76 ± 0.20	2.35 ± 0.20	2.33 ± 0.12
	150	4.99 ± 0.21	10.37 ± 0.17*	10.37 ± 0.17*	1.0*	–
Succinate (n = 4)	0	7.81 ± 1.03	41.42 ± 1.40	6.78 ± 0.48	6.21 ± 0.54	1.72 ± 0.07
	150	6.63 ± 1.06	23.58 ± 3.88*	10.07 ± 1.02*	2.11 ± 0.13*	1.35 ± 0.71
	300	6.70 ± 0.85	11.41 ± 1.03*	11.41 ± 1.03*	1.0*	–

The concentration of each substrate was 10 mM. Data are the means ± standard errors. Statistical significance relative to the controls is indicated by asterisks.

III respiration), however, quercetin reduced oxygen uptake with all substrates. Furthermore, the respiratory control was almost abolished, as can be judged from the state IV respiration rates and from the respiratory control ratios (state III/state IV). Under most conditions this action was so pronounced as to make it impossible to evaluate the ADP/O ratios using the traditional polarographic assay method [31].

Discussion

The basic conclusion allowed by the results is that quercetin decreases the mitochondrial NADH to NAD^+ ratio, as indicated by the reduced β-hydroxybutyrate to acetoacetate ratio in the presence of the compound. This action is consistent with previous reports about a similar reduction of the cytosolic NADH/NAD^+ ratio by quercetin [10]. This prooxidant activity [11] is probably a consequence of the reported ability of the compound to oxidize NADH when activated to *o*-semiquinones by peroxidases [1], [2], [12]. Hydrogen peroxide is always present in the liver, which possesses many enzymatic systems that are capable of producing free radicals from phenolic or polyphenolic compounds [13], [14], [15], [16]. A direct action on the mitochondrial enzyme β-hydroxybutyrate dehydrogenase is unlikely. Even at 300 μM, quercetin did not affect mitochondrial respiration dependent on β-hydroxybutyrate in the absence of ADP. In contrast, with 300 μM quercetin in the perfusion fluid the β-hydroxybutyrate to acetoacetate ratio was almost zero. In the presence of ADP, respiration of isolated mitochondria was inhibited with all substrates, suggesting a predominant action on the ATP-synthase [9]. It should also be emphasized that the β-hydroxybutyrate dehydrogenase is an enzyme that rapidly equilibrates β-hydroxybutyrate and acetoacetate [17]. For such enzymes, significant deviations from equilibrium occur only at very high inhibition degrees.

The action of quercetin on the mitochondrial NADH/NAD^+ ratio was already pronounced at relatively low quercetin concentrations (half-maximal action at 32.6 μM). It has been claimed that the use of quercetin in cancer treatment requires plasma concentrations between 10 and 100 μM because this is the range for which strongest tumor growth inhibitory effects of the compound have been reported [5]. There is thus a good overlap of the quercetin concentrations which can reduce the hepatic NADH/NAD^+ ratio and those which have antitumour effects. In humans such concentrations can only be achieved by means of endovenous administration [6], [32].

The action of quercetin on the NADH-NAD^+ redox potential was accompanied by a diversity of effects. The simultaneous oxygen uptake inhibition and Krebs cycle stimulation is an unusual combination of events that can be explained in terms of the reduced NADH to NAD^+ ratio. Stimulation of the Krebs cycle was most probably a consequence of the increased NAD^+ availability. The stimulating action of low NADH/NAD^+ ratios on the Krebs cycle is a well known phenomenon [18], [33]. Oxygen uptake inhibition by quercetin has probably two main causes: the diminished NADH concentration in the mitochondria and the inhibition of ADP phosphorylation. Stimulation of respiration in the liver by 25 μM quercetin is probably the consequence of the uncoupling action that superimposes on the inhibition of coupled respiration [9]. The reduced NADH/NAD^+ ratios are also likely to cause decreased biosynthetic activities which generally require considerable reducing power. The decreased reducing power in the presence of quercetin remains as the most likely explanation for the reported inhibition of hepatic gluconeogenesis [10].

In conclusion, it is likely that quercetin is able to induce a more oxidized state in the liver cells at concentrations that are required for it to exert therapeutic anti-cancer effects [5]. At least under the conditions of the isolated perfused rat liver, the prooxidant effects of quercetin seem to predominate over its antioxidant effects [5], [19]. Furthermore, especially in the liver, the prooxidant effects are possibly combined with other effects such as stimulation of glycogenolysis and glycolysis [9] and inhibition of gluconeogenesis [10]. In order to exert prooxidant effects, quercetin probably needs an enzymatic machinery able to generate *o*-semiquinones. There are many mammalian cell types that possess such enzymes. Leukocytes [34] and thyroid cells [35] are examples. It is thus possible that the prooxidant effects

of quercetin will be exerted in many types of cells so that this action probably extends over the whole organism.

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