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THE EFFECTS OF FISETIN ON NADH OXIDASE, SUCCINATE OXIDASE, ATPase ACTIVITIES AND TMPD/ASCORBATE OXIDATION

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ABSTRACT: Fisetin (3,7,3´,4´-tetrahydroxyflavone) is a flavonoid dietary ingredient found in fruits and vegetables. It exhibits a wide variety of pharmacological properties, including neurotrophic, antioxidant, anti-inflammatory, antidiabetic, antioangiogenic and anticarcinogenic effects. The most known biological effect of flavonoids is their antioxidant action. At high doses, however, the potentially toxic effects of fisetin and other flavonoids have to be taken into account, because it has been shown that in elevated doses, flavonoids, can act as mutagens, prooxidants with the generation of free radicals and as inhibitors of enzymes involved in energy and hormonal metabolism. The present work was planned to investigate the action of fisetin on specific points in the electron transport chain and ATPase activity of rat liver mitochondria. Male Wistar rats, weighing 200 to 280 g, fed with a standard laboratory diet were utilized. The ATPase activity of coupled mitochondria was increased in a dose-dependent manner; maximal stimulation of 134% was achieved at the concentration of 400 µM. When disrupted or uncoupled mitochondria were used as the enzyme source, the ATPase activity was inhibited by 43.6% and 36.7%, respectively, by fisetin 300 µM. The NADH oxidase activity was inhibited, but fisetin did not change the succinate oxidase activity and the TMPD/ascorbate oxidation. Fisetin affects electron flow as indicated by the inhibition of NADH oxidation in disrupted mitochondria probably by acting on the respiratory chain complex I, which is in accordance with previous study. Its effects on the ATPase activity suggest that fisetin is also an uncoupler of oxidative phosphorylation.

KEY WORDS: Fisetin; Flavonoids; Liver; Mitochondria.

1 INTRODUCTION

Fisetin (3,7,3´,4´-tetrahydroxiflavone) is a flavonoid dietary ingredient found in the smoke tree (*Cotinus coggyria*) and is also found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber at concentrations of 2 to 160 µg/g (ARAI et al., 2000). It exhibits a wide variety of pharmacological properties, including neurotrophic, antioxidant, anti-inflammatory and antioangiogenic effects. It has been reported to suppress the proliferation of tumor cells, such as prostate cancer, liver cancer, colon cancer, and leukemia. Certain bioflavonoids, including fisetin, inhibit glycolysis in Ehrlich ascites tumor cells (SUOLINNA et al., 1974; SUOLINNA et al., 1975).

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The most known biological effects of the flavonoids, including quercetin and fisetin, is no doubt their antioxidant action, which represents protection of tissues against the action of free-radicals and diminution of lipid peroxidation. However, the antioxidant and anticancer effects of quercetin and other flavonoids are not an unanimity, since it has been shown that significant protective effects due to quercetin would only be achieved upon the ingestion of high doses. At high doses, however, the potentially toxic effects of quercetin and other flavonoids have to be taken into account. It has been reported that flavonoids can act as mutagens, prooxidants with the generation of free radicals (CONSTANTIN; BRACHT, 2008) and as inhibitors of enzymes involved in energy metabolism and hormone actions (GASPARIN et al., 2003a,b).

The present work was planned to investigate if fisetin acts in some way on specific points of the chain carrier of electrons and also on the ATPse activity.

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 200 and 280 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, 2.0 mM Tris-HCl (pH 7.4), 0.2 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 50 mg% fatty acid-free bovine serum albumin. Homogenization was carried out in the same medium by means of a *Dounce* homogenizer. After homogenization, the mitochondria were isolated by differential centrifugation according to Voss et al (1961) using a sucrose-manitol isolation medium, and suspended in the same medium, which was kept at 0-4 ℃.

Rat liver mitochondria, isolated as described above, were disrupted by successive freeze and thawing procedures using liquid nitrogen and used as enzyme source for assaying membrane-bound enzymatic activities. NADH-oxidase and succinate-oxidase activities were assayed polarographically using a 20 mM TRIS-HCl (pH 7.4) medium. A polarographic assay was also run with TMPD (N,N,N',N'-tetramethyl-*p*-phenylenediamine) plus ascorbate as substrates. The reactions were started by the addition of 10 mM NADH, 10 mM succinate or 0.2 mM TMPD plus 5 mM ascorbate.

The ATPase activity was assayed by measuring phosphate release. When intact mitochondria were used as enzyme source, the reaction medium contained: 200 mM sucrose, 10 mM TRIS-HCl (pH 7.4), 50 mM KCl, 0.2 mM EGTA and, when required, 100 µM 2,4-dinitrophenol. 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 minutes of incubation at 37 ℃, by the addition of ice-cold 5% trichloroacetic acid. Phosphate was measured spectrometrically.

When coupled mitochondria were assayed, around 1 mg ml^{-1} of mitochondrial protein was used as enzyme source, and when uncoupled or disrupted mitochondria were assayed, around 0.5 mg ml^{-1} of mitochondrial protein was used as enzyme source. Protein contents of all experiments with mitochondria were measured using the method of Lowry et al (1951).

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

3 RESULTS AND DISCUSSION

The effects of several concentrations of fisetin on the NADH-oxidase activity, succinate-oxidase activity, and TMPD-ascorbate oxidation were measured in disrupted mitochondria and the mean values are shown in figure 1. The NADH-oxidase activity was inhibited in a dose-dependent manner. The mean concentration producing 50% inhibition (ID_{50}) of the NADH-oxidase activity was 242.80±20.32 µM**.** The maximal inhibition of the NADH-oxidase activity exceeded 82%. Neither succinate-oxidase activity nor TMPD-ascorbate oxidation were significantly affected.

The effects of fisetin on the ATPase activity were measured in intact mitochondria (coupled mitochondria), in the presence of 2,4-dinitrophenol (uncoupled mitochondria), and disrupted mitochondria, as shown in Figures 2 and 3. The ATPase activity of coupled mitochondria was increased, reaching a stimulation of about 120% with concentrations ranging from 200 µM to 600 µM. When disrupted or uncoupled mitochondria were used as the enzyme source, the ATPase activity was inhibited 43.6% ($p<0.05$) and 36.7% ($p<0.05$), respectively, by fisetin 300 µM.

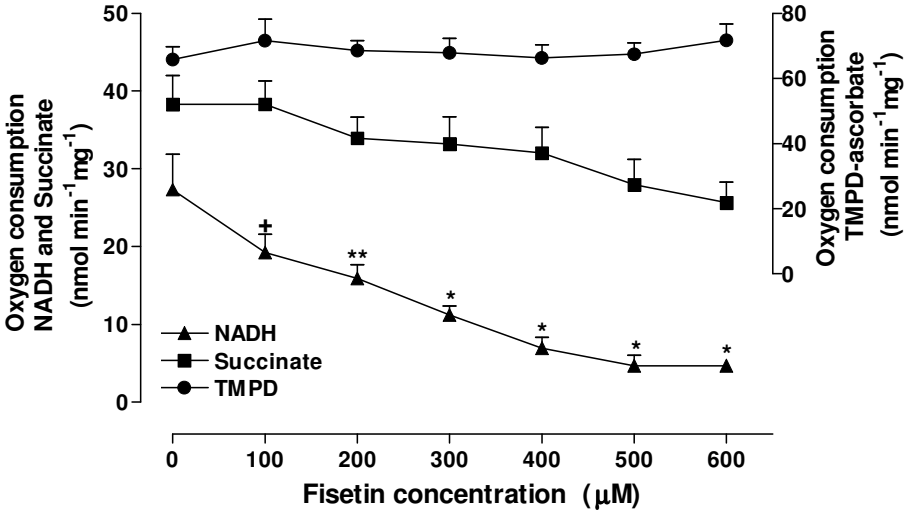

Figure 1. Effects of Fisetin on several membrane-bound enzymatic activities in rat liver mitochondria. NADH-oxidase, succinate-oxidase activities and TMPD-ascorbate oxidation were measured with freeze-thawing disrupted mitochondria, incubated at 37 ℃ in reaction medium as described in Materials and methods. Each data point is the mean ± SEM of six independent experiments. $^{+}p<0.05$, $^{**}p<0.01$, $^{*}p<0.001$, ANOVA with Newman-Keuls test.

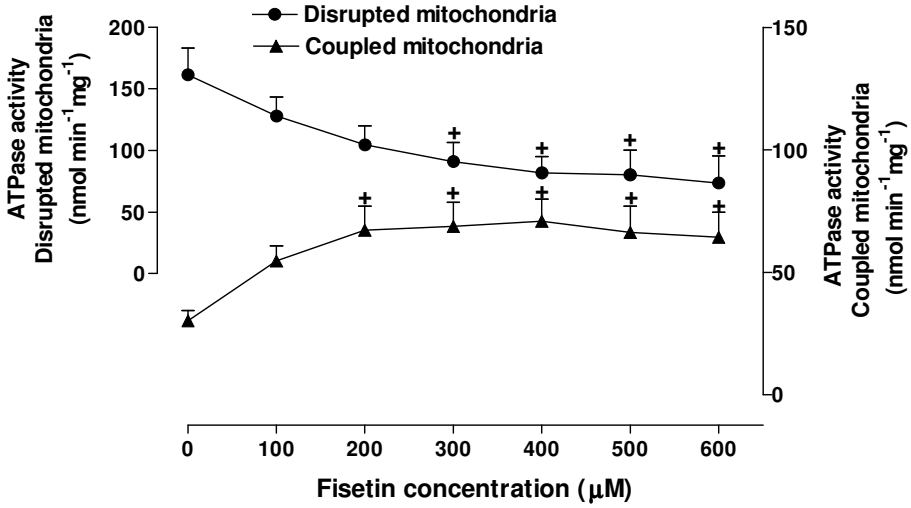

Figure 2. Effects of fisetin on the ATPase activity of coupled and disrupted mitochondria. The mitochondria were incubated at 37 °C in reaction medium as described in Materials and methods. Each assay point represents the mean of six (coupled mitochondria) and five (disrupted mitochondria) independent experiments. $^{+}p<0.05$, ANOVA with Newman-Keuls test.

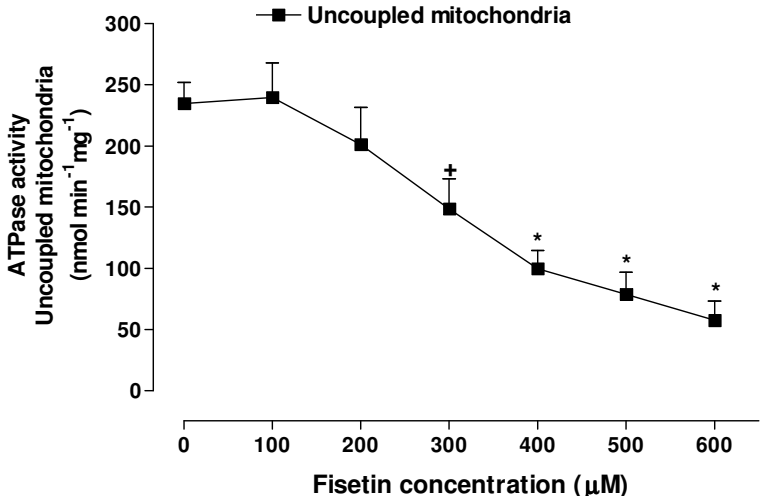

Figure 3. Effects of fisetin on the ATPase activity of uncoupled mitochondria. The mitochondria were incubated at 37 °C in reaction medium as described in Materials and methods. Each assay point represents the mean of six independent experiments. $^{+}p<0.05$, $^{*}p<0.001$, ANOVA with Newman-Keuls test.

4 CONCLUSION

The data of the present work show that fisetin is able to affect mitochondrial energy metabolism. Fisetin affects electron flow as indicated by the inhibition of NADH oxidation in disrupted mitochondria probably by acting on the respiratory chain complex I. An inhibitory action on ATP-synthase is indicated by the inhibition of the ATPase activity when disrupted or uncoupled mitochondria were used as the enzyme source. The ATPase activity of coupled mitochondria was increased, showing that fisetin acts as uncoupler of oxidative phosphorylation.

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