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Eupafolin: Effect on mitochondrial energetic metabolism☆

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Abstract—This study evaluated the effects of flavone eupafolin (6-methoxy 5,7,3′,4′-tetrahydroxyflavone), extracted from dry leaves of *Eupatorium litoralle*. Eupafolin (25–200 μM) promoted inhibition of the respiratory rate in state 3, in the presence of glutamate or succinate. During succinate oxidation, it was found that only state 4 respiratory rate was stimulated ~30% by eupafolin (100 μM) and ADP/O ratio and RCC were reduced with all doses. When glutamate was used as substrate, RCC was similarly reduced. Eupafolin caused a reduction of enzymatic activities between complexes I and III of the respiratory chain. Cytochrome *c* oxidase and ATPase activities were not affected. Using voltammetry cyclic analysis, eupafolin give rise to irreversible oxidation with an anodic peak potential at +0.08 V (SHE). We also observed that eupafolin can undergo oxidation catalyzed by EDTA–Fe, promoting cytochrome *c* reduction in the presence of NADH, resulting in the production of the superoxide radical and hydrogen peroxide. All together, the results could explain the cytotoxic effects observed previously with the eupafolin.

1. Introduction

Flavonoids are a class of secondary plant phenolics of low molecular weight,[1] being derivatives of benzo-γ-pyrone,[2] distributed in all vascular plants[3] and present in most edible fruits and vegetables of the human diet.[4]

Many flavonoids are able to act as antioxidants through a free radical scavenging mechanism, transferring electrons to free radicals with the formation of less reactive flavonoid phenoxyl radicals. Their antioxidant effect can also be caused by their ability to chelate transition metals.[5–8] Antioxidants may protect cells against free radicals that promote cellular damage involved in different disorders, including ischemic conditions and tumor development.[9] They possess a remarkable spectrum of other biological activities, affecting cell functions such as growth, differentiation, and apoptosis.[10–13]

Some flavonoids contain a catechol ring, a characteristic that has been observed in compounds that can undergo autooxidation, generating reactive oxygen species (e.g., catecholamines)[14] making them capable of acting as prooxidants.[15]

Structure–activity studies on flavonoids demonstrated that the inhibition of succinate oxidase appears to be linked to their abilities to participate in oxidation–reduction reactions and that the most potent inhibitors possess hydroxyl substituents configurations, namely, hydroquinone and catechol.[16] Inhibition of mitochondrial enzymes by flavonoids may contribute to their cytotoxic and antineoplastic activities.[17,18]

Standard reduction potentials of individual electron carriers of the mitochondrial respiratory chain have been determined and vary from −0.320 to +0.816 V. Hence it is important to verify the reduction potential of flavonoids and if some of the biological activities may be dependent on their direct action on electron flow in the respiratory chain.

Eupafolin (6-methoxy 5,7,3′,4′-tetrahydroxyflavone) (Fig. 1), the flavone used in this study, was extracted from *Eupatorium litoralle*. Some medicinal plant extracts

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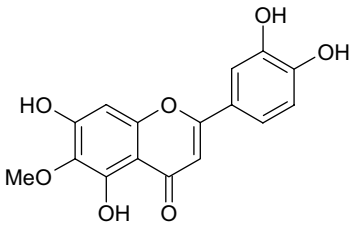

Figure 1. Structure of eupafolin (6-methoxy 5,7,3′,4′-tetrahydroxyflavone).

used in Brazil also contain this flavone.[19–24] Eupafolin possesses several known biological properties, among them: (i) it promotes iron release from ferritin, and donates electrons to the stable free radical DPPH[25]; (ii) it protects cultured neurons against glutamate-induced oxidative stress[12]; and (iii) it inhibits xanthine oxidase activity.[26] Eupafolin also has antiproliferative activity against MK-1 (human gastric adenocarcinoma), B16-F10 (murine melanoma), and HeLa (human uterine carcinoma)[5] cells. Although some effects of eupafolin have been demonstrated, its mechanism of action is not well known. The effects of eupafolin on mitochondrial metabolism and redox properties were evaluated, in order to contribute to the understanding of its cytotoxicity mechanism, especially on mitochondrial metabolism.

2. Materials and methods

2.1. Chemicals

Glutamic acid, succinic acid, NADH, ATP, ADP, EGTA, EDTA, FCCP, DPPH, rotenone, D-mannitol, sucrose, Hepes, BSA, phosphoenolpyruvate (PEP), pyruvate kinase, valinomycin, oligomycin, cytochrome *c*, and Tris were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, ammonium heptamolybdate, and ferrous sulfate were purchased from Merck (Brazil). Other reagents were of analytical grade. Solutions were prepared with Millipore Milli Q deionized water.

2.2. Animals

The animals used were male albino rats (Wistar strain; 220–300 g), which received a standard laboratory diet (Nuvilab®) and water ad libitum. All animals were starved for 12 h prior to being sacrificed, and none of then showed any pathological lesion. All recommendations of the Brazilian Law (No. 6.638.05 November 1979) for scientific management of animals were respected.

2.3. Preparation of eupafolin solutions

Eupafolin was extracted from leaves of *Eupatorium litoralle* and its structure confirmed as described.[27] After purification by HPLC, eupafolin purity was checked by HPLC at $\lambda = 339$ nm by injecting the collected fraction and just one peak was observed in the chromatogram. It was dissolved in DMSO and then further diluted with the assay medium. Solvent controls with DMSO were carried out in each assay. Flavone stock solution was stored at 4 °C and brought to 25 °C before use.

2.4. Preparation of rat liver mitochondria

Rat liver mitochondria were prepared according to the procedure of Voss et al.[28] with slight modifications. Male Wistar rats were sacrificed by decapitation; livers were immediately removed, sliced in extraction medium containing D-mannitol (210 mM), sucrose (70 mM), Tris–HCl buffer (10 mM) (pH 7.4), EDTA (0.5 mM), and BSA (0.05 g%), and homogenized three times with a Potter–Elvehjem homogenizer. Homogenates were centrifuged at 2500*g* for 5 min and the resulting supernatant was further centrifuged at 12,500*g* for 10 min. Pellets were suspended in medium and centrifuged twice at 10,000*g*. The final mitochondrial pellet was suspended in 0.5 mL of medium. For the polarographic experiments, only mitochondrial preparations with RCC $\geqslant$ 4.0 were used. To evaluate the enzyme complexes of the respiratory chain, mitochondrial suspensions were frozen in liquid N_2 and at the time of use, each aliquot was disrupted by a freeze-thaw treatment (×3) and maintained at 4 °C during the enzymatic tests.

2.5. Oxygen uptake

Oxygen uptake and oxidative phosphorylation were carried out using a Clark-type oxygen electrode (Yellow Springs Instruments Co.), with a Gilson Oxygraph, and evaluated at 30 °C in a 1.3 mL thermostatically controlled, water jacketed, closed chamber with magnetic stirring. A reaction medium containing D-mannitol (125 mM), KCl (65 mM), Hepes–KOH buffer (pH 7.2) (10 mM), EGTA (0.1 mM), and BSA (0.1 g%) was supplemented with sodium succinate (2.5 mM), rotenone (10 μM), Pi (0.8 mM), ADP (0.16 mM), and mitochondrial protein (0.5 mg), or with sodium glutamate (5 mM), KH_2PO_4 (1.6 mM), ADP (0.16 mM), and mitochondrial protein (2 mg). Eupafolin was added to each system at varying concentrations (0.025–0.2 mM). The respiratory rate is expressed as nmol of oxygen consumed per minute per milligram of mitochondrial protein, according to Estabrook.[29] The ADP/O ratio was calculated according to Chance and Williams.[30] RCC values were obtained as the ratio between the rate of mitochondrial oxygen consumption in state 3 and the rate of mitochondrial oxygen consumption in state 4.

2.6. Measurement of activities of enzyme complexes of the respiratory chain

NADH oxidase and succinate oxidase were assayed by polarographic determinations, NADH-dehydrogenase (NADH: ubiquinone oxidoreductase) and succinate-dehydrogenase as described by Singer.[31] NADH–cytochrome *c* reductase (NADH: cytochrome *c* oxidoreductase) and succinate–cytochrome *c* reductase (succinate: cytochrome *c* oxidoreductase) activities were measured by reduction of cytochrome *c* at 550 nm as described by Somlo.[32] The activity of cytochrome *c* oxidase was

determined at 550 nm, according to Mason et al.[33] ATPase activity in disrupted and intact mitochondria was evaluated as described by Pulmann et al.[34] but with modifications. In disrupted mitochondria the enzyme was assayed at 37 °C, using a medium consisting of sucrose (250 mM), Tris–HCl (50 mM) buffer (pH 8.4), magnesium sulfate (3.0 mM), PEP (2.5 mM), pyruvate kinase (10 U), ATP (4 mM), and mitochondrial protein (100 μg). Results are expressed as nmol of Pi liberated per min per mg. The ATPase activity of intact mitochondria was assayed at 30 °C in the presence or absence of FCCP (1 μM), the reaction medium consisting of sucrose (50 mM), Tris–HCl (120 mM) buffer (pH 7.4), KCl (50 mM), ATP (3 mM), and mitochondrial protein (1 mg).

2.7. Cyclic voltammetry

Cyclic voltammogram was recorded at 25 °C in Tris–HCl (10 mM) buffer, pH 7.4, and EGTA (0.1 mM). An EG & PAR (Princeton Applied Research) electrochemistry apparatus, model 173A, was used. The working electrode was a glassy carbon disk. The auxiliary electrode was a platinum wire and the reference electrode was a silver (Ag/AgCl) electrode. To prevent possible atmospheric oxidation of compounds over the physiological pH range, oxygen-free nitrogen was used to thoroughly purge and blanket the analyzed solutions, before recording the voltammogram. The rate of scans of the potentials was kept constant at 50 mV s^{-1}.

2.8. Reduction of cytochrome *c* in the presence of NADH, EDTA, and eupafolin

The reduction of cytochrome *c* was measured using a HITACHI (mod. U-2001 UV/VIS) spectrophotometer. The reaction medium contained pH 7.4 phosphate buffer (50 mM), EDTA (2 mM), cytochrome *c* (40 μM), NADH (50 μM), superoxide dismutase (80 U/mL), desferoxamine (0.5 mM), catalase (25 U), and eupafolin (200 μM). The reactions were carried out at 28 °C in a final volume of 1.0 mL and accompanied through absorbance variation of cytochrome *c* reduction at 550 nm.

2.9. Protein determination

Mitochondrial protein was assayed by the method of Lowry et al.[35] calibrated with bovine serum albumin.

2.10. Statistical analysis

Data are presented as means ± SD. Statistical analysis of the data was carried out as analysis of variance, linear regression and Tukey test for average comparison. Results were considered significant when $p < 0.05$.

3. Results

3.1. Effects of eupafolin on mitochondrial oxygen uptake

To investigate the mechanism of action of eupafolin on mitochondrial functions, we assessed its effect on isolated rat liver mitochondria. The rates of oxygen consumption on addition of ADP (state 3) and after its exhaustion (state 4) in the presence of eupafolin (25–200 μM), the respiratory control coefficient (RCC), and the ADP/O ratio were measured. Tables 1 and 2 show the effects of eupafolin on these parameters when succinate or glutamate was the oxidizable substrate, respectively. With succinate, the respiratory rate in state 3 was inhibited by ~17, ~45, ~50, ~55, and ~65% (Table 1) and with glutamate by ~15, ~34, ~45, ~68 and 80% (Table 2) in the presence of 25, 50, 75, 100, and 200 μM of eupafolin, respectively. The results demonstrate a linear dose-dependent decrease of oxygen consumption in state 3 verified in the presence of 50–200 μM of eupafolin when succinate was used as substrate ($r^2 = 0.975$) and 25–100 μM when glutamate was used as substrate ($r^2 = 0.983$).

The state 4 respiratory rates (after ADP consumption) were not affected by eupafolin at doses under 100 μM for both substrates. But at 100 μM of eupafolin the oxidation of succinate was stimulated by ~30% (Table 1) and in the presence of 200 μM, the mitochondrial respiration did not return to state 4 after ADP phosphorylation (data not shown), indicating uncoupling of respiration from phosphorylation at high doses of eupafolin. The RCC values were decreased with both substrates at all tested concentrations of the flavone. Linear dose-dependent decrease was observed in the presence of eupafolin (25–100 μM) when glutamate ($r^2 = 0{,}957$) or succinate ($r^2 = 0.922$) was used as substrate. It (100 μM) caused inhibition of ~76% and 63% of the respiratory control, with glutamate and succinate, respectively, and decreased the ADP/O ratio by 25% when succinate was the substrate. However, the presence of eupafolin did not change the ADP/O ratio, when glutamate was the substrate. The results show that its effect on RCC is similar using substrates for complexes I (glutamate) and II (succinate), unlike the effect on the ADP/O ratio which only in the presence of succinate is decreased to some extent. These differences suggest uncoupling of mitochondria caused by high doses of eupafolin when respiring on succinate, added to a decrease in the phosphorylation potential in the presence of the substrate for complex I (glutamate), only an effect on the phosphorylation potential was evidenced. In addition, it was verified that eupafolin (75–200 μM) reduced the oxygen consumption when succinate was used as the substrate in mitochondria uncoupled with FCCP (data not shown). These data suggest inhibitory effect of eupafolin on the respiratory chain.

3.2. Eupafolin effects on enzyme activities

Flavonoids have been reported to promote inhibition of enzyme complexes in the respiratory chain.[16] The inhibition promoted by eupafolin on oxygen uptake motivated us to study its effects on enzyme activities of the complexes in the respiratory chain. The effects of eupafolin on enzyme activities are listed in Table 3. It promoted inhibition in a dose-dependent manner of NADH oxidase and at the highest concentration

Table 1. Effect of eupafolin on respiratory parameters using succinate as oxidizable substrate

Eupafolin (μM)	State III (%)	State IV (%)	RCC (%)	ADP/O (%)
0	100	100	100	100
25	83.0 ± 7.3*	105.7 ± 15.9	76.4 ± 9.0*	85.8 ± 5.8*
50	55.2 ± 7.3*	108.6 ± 13.8	55.1 ± 7.2*	85.4 ± 6.1*
75	51.2 ± 6.8*	121.0 ± 19.4	52.9 ± 7.4*	81.6 ± 9.1*
100	45.4 ± 11.8*	131.5 ± 26.0*	36.8 ± 4.5*	74.7 ± 8.9*
200	34.7 ± 3.4*	—	—	—

Conditions for oxygen uptake measurements are described in Section 2. Control (100%) corresponds to: 57 ± 13.6 nmol of oxygen consumed min^{-1} mg^{-1} protein in state III, 11.8 ± 0.7 nmol of oxygen consumed min^{-1} mg^{-1} protein in state IV, 2.3 ± 0.3 nmol of oxygen consumed min^{-1} mg^{-1} protein in ADP/O ratio, and RCC 4.3 ± 0.4. Each value represents median ± SD of four different experiments.
* Significantly different from control ($p \leqslant 0.05$).

Table 2. Effect of eupafolin on respiratory parameters using sodium glutamate as oxidizable substrate

Eupafolin (μM)	State III (%)	State IV (%)	RCC (%)	ADP/O (%)
0	100	100	100	100
25	84.9 ± 10.8*	108.9 ± 11.7	73.0 ± 6.1*	92.8 ± 6.4
50	66.0 ± 9.2*	102.3 ± 9.3	66.5 ± 10.1*	85.0 ± 7.9
75	54.9 ± 11.8*	109.1 ± 15.3	40.9 ± 5.7*	90.6 ± 6.9
100	31.8 ± 6.4*	99.4 ± 18.7	24.9 ± 5.0*	88.6 ± 13.5
200	19.8 ± 2.0*	—	—	—

Conditions for oxygen uptake measurements are described in Section 2. Control (100%) corresponds to: 38 ± 5.4 nmol of oxygen consumed min^{-1} mg^{-1} protein in state III, 8.5 ± 0.7 nmol of oxygen consumed min^{-1} mg^{-1} protein in state IV, 3.0 ± 0.4 nmol of oxygen consumed min^{-1} mg^{-1} protein in ADP/O ratio, and RCC 4.6 ± 0.5. Each value represents median ± SD of four different experiments.
* Significantly different from control ($p \leqslant 0.05$).

Table 3. Effects of eupafolin on enzyme activities of the mitochondrial respiratory chain

Eupafolin (μM)	NADH oxidase (%)	NADH-dehydrogenase (%)	NADH–cytochrome *c* reductase (%)	Succinate oxidase	Succinate dehyidrogenase (%)	Succinate–cytochrome *c* reductase (%)	Cytochrome *c* oxidase (%)
0	100	100	100	1000	100	100	100
25	80 ± 5*	42 ± 3*	54 ± 8*	87 ± 15	71 ± 11*	41 ± 5*	98 ± 15
50	64 ± 8*	41 ± 4*	45 ± 9*	83 ± 13	69 ± 10*	23 ± 5*	104 ± 10
75	50 ± 6*	51 ± 7*	46 ± 8*	86 ± 15	78 ± 8*	12 ± 2*	102 ± 11
100	41 ± 4*	50 ± 7*	40 ± 5*	77 ± 11*	50 ± 3*	29 ± 5*	96 ± 9
200	24 ± 1*	39 ± 6*	19 ± 2*	77 ± 9*	51 ± 8*	18 ± 1*	82 ± 10

Experimental conditions are described in Section 2. Control values (100%) were: NADH oxidase: 42 ± 6.7 nmol of O_2 consumed min^{-1} mg^{-1} of mitochondrial protein; NADH citochrome *c* reductase: 27.4 ± 5.5 nmol of cytochrome *c* reduced. min^{-1} mg^{-1} of mitochondrial protein; NADH-dehydrogenase: 24.22 ± 3.9 μmol of ferricyanide reduced min^{-1} mg^{-1} of mitochondrial protein. Succinate–cytochrome *c* reductase: 21.4 ± 6 nmol of cytochrome *c* reduced. min^{-1} mg^{-1} of mitochondrial protein; succinate dehydrogenase: 149 ± 6 μmol of DCPIP reduced. min^{-1} mg^{-1} of mitochondrial protein; cytochrome *c* oxidase: 601 ± 8 μmol cytochrome *c* oxidized min^{-1} mg^{-1} of mitochondrial protein. Results (means ± SD of four independent experiments in triplicate) are expressed as % of control activities. Results (means ± SD of three independent experiments) are expressed as % of control activities.
* Significantly different from the control (100%), $p < 0.05$.

(200 μM), the inhibition was 76%. These results are in agreement with the inhibition verified in state 3 respiration, with glutamate as substrate (Table 2). At the same dose, NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were inhibited by ~80% and NADH-dehydrogenases and succinate-dehydrogenases by ~60% and ~50%, respectively. Succinate oxidase was inhibited (~25%) only with 100 and 200 μM of eupafolin. Cytochrome *c* oxidase (Table 3) and ATPase activities using intact mitochondria in the presence or absence of FCCP or disrupted mitochondria were not affected (Table 4). The enzyme complex analyses showed that eupafolin caused inhibition of activities between complexes I and III.

Table 4. Effects of eupafolin on enzymatic activities of the ATPases

Eupafolin (μM)	ATPase disrupted (%)	ATPase intact (%)	ATPase intact with FCCP (%)
0	100	100	100
25	103 ± 14	99 ± 18	93 ± 13
50	102 ± 11	94 ± 6	90 ± 14
75	105 ± 12	98 ± 15	97 ± 7
100	94 ± 8	97 ± 13	100 ± 13
200	90 ± 15	101 ± 7	102 ± 10

Experimental conditions are described in Section 2. Control values (100%) were, ATPase intact: 34.2 ± 9 μmol Pi liberated; ATPase with FCCP: 64 ± 5 μmol Pi liberated. Results (means ± SD of four independent experiments in triplicate) are expressed as % of control activities. Results (means ± SD of three independent experiments) are expressed as % of control activities.

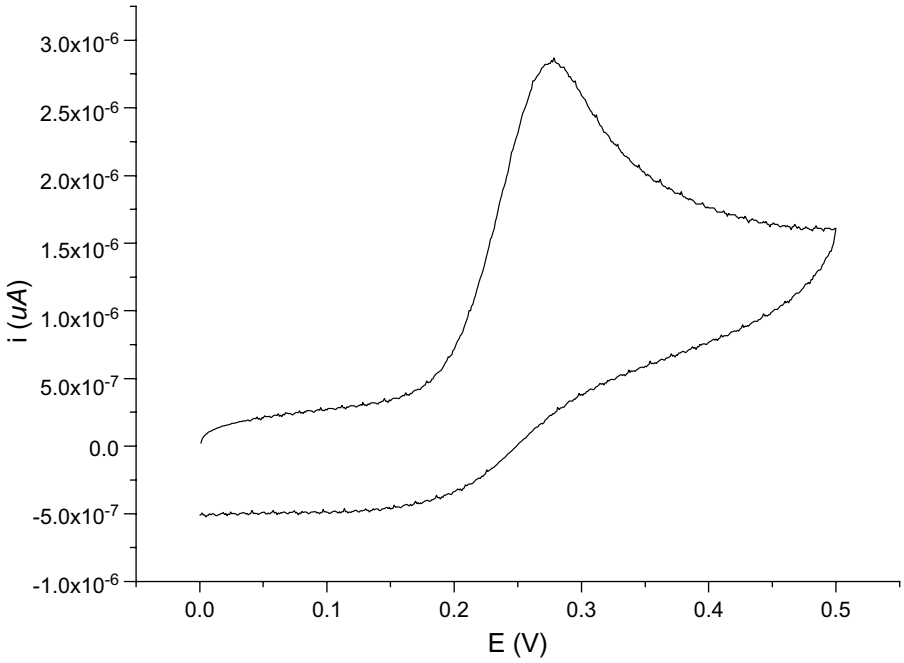

Figure 2. Cyclic voltammogram of eupafolin.

3.3. Electrochemical properties of eupafolin

In order to investigate whether the inhibition of enzyme activity in the respiratory chain is linked to the enzyme's ability to participate in oxidation–reduction reactions, cyclic voltammetry analyses were performed. Eupafolin suffered irreversible oxidation with the anodic peak potential at +0.28 V (Ag/AgCl) (Fig. 2). The conversion to a hydrogen standard electrode resulted in a potential value of +0.08 V (SHE), indicating that this flavone oxidizes at potential values observed in the mitochondrial respiratory chain. The results suggest that eupafolin, which possesses a catechol ring, could undergo oxidation, generating possible semiquinones and free radicals.

3.4. Eupafolin reduction of cytochrome *c*

Considering that eupafolin is able to inhibit enzyme activities between complexes I and III in the mitochondrial respiratory chain and that it undergoes oxidation at a potential value at +0.08 V (SHE), a hypothesis was formulated that this flavone interferes directly on electron transport. It is important to emphasize that compounds containing a catechol ring (6-hydroxy-dopamine) in their structure undergo autooxidation stimulated by addition of metal ions, generating reactive oxygen species (ROS) and semiquinones.[14] Given that eupafolin contains this group in its structure, the question also arised as to whether free radicals are generated during its oxidation, which could react directly with electron carriers, such as cytochrome *c*. Figure 3 shows the effect of eupafolin on cytochrome *c* reduction in the presence and absence of NADH, EDTA, and antioxidant enzymes to verify ROS generation. Cytochrome *c* reduction was observed in the presence of NADH and eupafolin. When EDTA was added, cytochrome *c* reduction was increased (Fig. 3). These results suggest involvement of iron ions in this process and the importance of NADH presence in the system. Small amounts of iron are sufficient to promote ROS generation capable of reducing cytochrome *c*. One of these ROS could be $O_2^{\cdot-}$. In fact, cytochrome *c* reduction was lower when SOD (~46%) or catalase (~15%) was added (Fig. 3). Since these enzymes decompose $O_2^{\cdot-}$ and H_2O_2, respectively, these results suggest the possible generation of these ROS during the process. In the presence of both enzymes, the reduction of cytochrome *c* was similar to that obtained with catalase or deferoxamine alone (data not shown). These confirm the importance of the presence of iron in ROS generation, since EDTA raised and deferoxamine lowered cytochrome *c* reduction. It also suggests that eupafolin undergoes oxidation in the presence of the metal and NADH, probably generating semiquinones, superoxide radicals, and H_2O_2 during cytochrome *c* reduction.

4. Discussion

Flavonoids have won recent interest because of their broad pharmacological activity. Putative therapeutic effects of many traditional medicines may be ascribed to the presence of flavonoids. The pharmacological effect of flavonoids is especially due to their inhibition of certain enzymes and their antioxidant activity.[36–38] Flavonoids possess well-recognized antioxidant and prooxidant properties.[39] Eupafolin has some biological effects, such as cytotoxicity to tumoral cells. Many flavonoids are capable of inhibiting enzyme activities in the mitochondrial respiratory chain[16,17] or undergoing redox reactions, and these effects are related to their cytotoxicity. The mechanism of action of the eupafolin and its redox properties are not well known. Compounds that have catechol ring in its structure, such as the

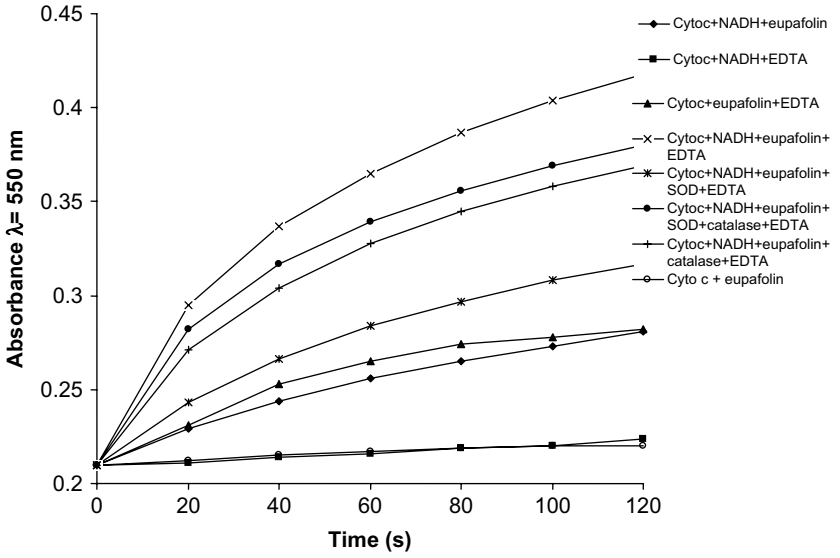

Figure 3. Effect of eupafolin on reduction of cytochrome *c*.

eupafolin, are capable of undergoing oxidation generating reactive oxygen species (ROS)[14] and promoting cell damage. Thus we directed this study to verify whether eupafolin promotes alterations in mitochondrial metabolism and if it can suffer autooxidation generating reactive oxygen species that might be involved with the cytotoxicity observed with eupafolin.

It was verified that eupafolin promoted an intense inhibition in the rate of oxygen consumption at state 3, using glutamate or succinate as oxidizable substrates, and decreased the ADP/O ratio and RCC (Tables 1 and 2). These results are in agreement with those of Coleman et al.[40] who used only a single dose of eupafolin (40 μM), and verified inhibition in state 3 when glutamate and succinate were used as substrates. Eupafolin did not affect ATPase activity. These results are similar to those observed previously in our laboratory with hispidulin (6-methoxy 5,7,4′-trihydroxyflavone), a flavone similar to eupafolin, except for the absence of the HO—3′ group. Hispidulin (50–200 μM) decreased the respiratory rate in state 3, the ADP/O ratio, and RCC, but stimulated the respiratory rates in state 4 at doses from 75 to 200 μM and inhibited state 3 respiration when succinate was the substrate. However, when mitochondria were respiring using glutamate, a stimulus in the respiratory rate in state 4 was observed only at doses of 150 and 200 μM of hispidulin and the inhibition in the respiratory rate in state 3 was also observed. These results suggest that these values of stimulation might be different with succinate or glutamate as substrate, probably because the sequence of electron carriers used in the mitochondrial respiratory chain is different for these substrates.

The decrease in oxygen uptake is related to the inhibitions caused by eupafolin on enzyme activities of electron transport in the mitochondrial respiratory chain (Table 3). The results on enzymatic activities indicated that the main eupafolin inhibition site is between complexes I and III and that it did not affect ATPase activity. These results are in accordance with those observed for hispidulin, which also inhibited enzyme activities between complexes I and III. Our results confirm that the presence of the C-4 keto group and the $C_{2,3}$ double bond is a structural feature of flavonoids that are important for inhibition of NADH oxidase.[16]

The effects of eupafolin on the state 3 respiratory rate and on enzyme activities in the respiratory chain were greater than with hispidulin. These results are possibly related to the structural differences of eupafolin and its redox properties, because electrochemical analyses demonstrated that it undergoes irreversible oxidation in +0.08 V (SHE), probably on the catechol ring. These results are in agreement with those of Hodnick and colleagues.[18]

The results observed in the absence of enzymes demonstrated that eupafolin can undergo autooxidation, promoting cytochrome *c* reduction in the presence of NADH and iron. The autooxidation probably occurs in the catechol moiety of its ring B, as demonstrated for other phenols such as 6-hydroxy-dopamine, generating free radicals.[14] The effects attributed to ROS generation could be confirmed when SOD and catalase were added and a decrease of cytochrome *c* reduction was observed, indicating the involvement of $O_2^{\cdot-}$ and H_2O_2 in the system. Our hypothesis is that, during eupafolin autooxidation, $O_2^{\cdot-}$ and semiquinone (SQ) are generated (1).

$$\text{Eupafolin} + O_2 \rightarrow \text{SQ(Eupafolin)}^{\cdot} + O_2^{\cdot-} \quad (1)$$

The semiquinone, in the presence of oxygen, can be converted to quinone (Q) and the superoxide radical (2) or the semiquinone can react with $O_2^{\cdot-}$, generating the quinone and hydrogen peroxide (3).

$$\text{SQ(Eupafolin)}^{\cdot} + O_2 \rightarrow \text{Q(Eupafolin)} + O_2^{\cdot-} \quad (2)$$

$$\text{SQ(Eupafolin)}^{\cdot} + O_2^{\cdot-} \rightarrow \text{Q(Eupafolin)} + H_2O_2 \quad (3)$$

Besides, eupafolin can react with $O_2^{\cdot-}$ generating the semiquinone and H_2O_2 (4).

$$\text{Eupafolin} + O_2^{\cdot-} \rightarrow \text{SQ(Eupafolin)}^{\cdot} + H_2O_2 \quad (4)$$

The $O_2^{\cdot-}$ generated in reactions can promote cytochrome *c* reduction (5). If this is the case, addition of SOD and/or catalase would suppress the cytochrome *c* reduction, as it was observed in our results (Fig. 3).

$$\text{Cytochrome } c + O_2^{\cdot-} \rightarrow \text{Cytochrome } c \text{ reduced} + O_2 \quad (5)$$

Since the presence of EDTA increased the rate of reduction of cytochrome *c* and deferoxamine decreased it, we concluded that divalent iron was important in this process. The traces of the metal in the buffer suffered oxidation, generating $O_2^{\cdot-}$ (6).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-} \quad (6)$$

NADH addition promoted an increase in the rate of reduction of cytochrome *c*, probably because the semiquinone of eupafolin, generated during autooxidation, would react with NADH, generating $NAD^{\cdot}$ and contributing to cytochrome *c* reduction as represented in reactions (7) and (8).

$$\text{NADH} + \text{SQ(Eupafolin)}^{\cdot} \rightarrow NAD^{\cdot} + H^{+} + \text{Eupafolin} \quad (7)$$

$$NAD^{\cdot} + \text{Cytochrome } c \rightarrow \text{Cytochrome } c \text{ reduced} + NAD^{+} \quad (8)$$

These observations together with the results previously reported for eupafolin on iron released from ferritin[25] could contribute to explain the cytotoxic effects in cells.

5. Conclusion

Eupafolin, a flavone present in plants, promotes an intense inhibitory effect on the oxygen consumption rate in mitochondria isolated from rat liver. We believe that this effect occurs due to modifications in the mitochondrial respiratory chain, mainly between complexes I and III.

This flavonoid also suffers oxidation at a potential range of the respiratory chain, and promoted the reduction of cytochrome *c* in a non-enzymatic way, depending on the presence of NADH and iron to generate ROS which could contribute to explain the effects on the mitochondrial metabolism.

It is important to consider also that mitochondria are potent producers of cellular superoxide, from complexes I and III of the electron transport chain, and that with eupafolin inhibitions in these enzymatic activities, ROS generation could be modified. Superoxide production is very sensitive to the proton motive force, so it can also be strongly decreased by mild uncoupling.

Accordingly, a structure–activity study employing a series of model phenolic compounds demonstrated that the most potent succinate oxidase inhibitors possessed hydroxyl configurations capable of supporting oxidation–reduction reactions (i.e., hydroquinone and catechol).[16]

Our hypothesis is that the inhibitory effect of eupafolin on oxygen consumption is caused by its interaction with the mitochondrial respiratory chain components, inhibiting enzyme activities and also by generating ROS during eupafolin oxidation via non-enzymatic reactions. We cannot ignore the possibility of direct interference of eupafolin on electron transport, especially in complexes in which cytochrome *c* is present.

The effects on mitochondrial function could play a role in the cytotoxicity of eupafolin observed by other authors and indicates the importance of the catechol ring in promoting these effects.

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