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Complex I and cytochrome *c* are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria

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ABSTRACT

Flavonoids can protect cells from different insults that lead to mitochondria-mediated cell death, and epidemiological data show that some of these compounds attenuate the progression of diseases associated with oxidative stress and mitochondrial dysfunction. In this work, a screening of 5 flavonoids representing major subclasses showed that they display different effects on H_2O_2 production by mitochondria isolated from rat brain and heart. Quercetin, kaempferol and epicatechin are potent inhibitors of H_2O_2 production by mitochondria from both tissues ($IC_{50} \approx 1–2$ μM), even when H_2O_2 production rate was stimulated by the mitochondrial inhibitors rotenone and antimycin A. Although the rate of oxygen consumption was unaffected by concentrations up to 10 μM of these flavonoids, quercetin, kaempferol and apigenin inhibited complex I activity, while up to 100 μM epicatechin produced less than 20% inhibition. The extent of this inhibition was found to be dependent on the concentration of coenzyme Q in the medium, suggesting competition between the flavonoids and ubiquinone for close binding sites in the complex. In contrast, these flavonoids did not significantly inhibit the activity of complexes II and III, and did not affect the redox state of complex IV. However, we have found that epicatechin, quercetin and kaempferol are able to stoichiometrically reduce purified cytochrome *c*. Our results reveal that mitochondria are a plausible main target of flavonoids mediating, at least in part, their reported preventive actions against oxidative stress and mitochondrial dysfunction-associated pathologies.

1. Introduction

It is widely accepted that mitochondrial production of reactive oxygen species (ROS) contributes to the detrimental alterations in the etiology and/or progression of many pathological conditions, including neurodegeneration, brain and myocardial ischemia–reperfusion injury, vascular disease and heart failure (for reviews, see [1–5]).

Production of ROS by mitochondria is stimulated by the types of inhibition of respiratory chain observed in some cardiac diseases [1] and in Parkinson's and Huntington's brain [2,6], as well as by several cellular signaling pathways activated in different degenerative processes, e.g. tumor necrosis factor-α, oncogenes, hypoxia and nitric oxide [7,8].

The complexes I and III of the mitochondrial respiratory chain are known to be major sources of intracellular superoxide anion [2,9], and cellular oxidative stress has been shown to mediate the neurodegeneration of critical brain areas observed in ischemia–reperfusion insults [10,11], inflammation [12–14], neurotoxicity of drugs and environmental chemicals [15–18], and also in Alzheimer, Parkinson and other neurodegenerative diseases [19–22]. Therefore, substances or therapies targeting basic mitochondrial processes, such as energy metabolism or free radical generation hold great promise for disease prevention and treatment.

Flavonoids are low molecular weight phenolic compounds with a quinonoid-like chemical structure, which display significant ROS scavenging and metal chelating activities, as well as other cellular antioxidant actions [23–25]. Because of the implication of ubiquinone radicals in mitochondrial ROS production [26], mitochondrial respiratory chain components can be seen as likely molecular targets for the cellular antioxidant actions of flavonoids. The data from dietary bioavailability of flavonoids point out that physiologic plasma concentrations of total metabolites do not exceed 10 μmol/

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl ether) N,N,N′,N′-tetraacetic acid; IC_{50}, 50% inhibitory concentration; ROS, reactive oxygen species; SOD, superoxide dismutase; Tris, tris-(hydroxymethyl)aminomethane

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L [27]. The bioavailability of flavonoids also depends on the dietary sources, and it has been shown that total flavonoid metabolites can reach plasma concentrations up to 4.0 μmol/L with an intake of 50 mg aglycone equivalents, except for isoflavones that can reach higher concentrations [28]. There is substantial evidence from experiments with animal models that flavonoids can access the brain. After oral administration several flavonoids have been detected in rat and mouse brains, like epigallocatechin gallate and epicatechin metabolites [29,30], quercetin and quercetin metabolites [31] and anthocyanidins [32,33]. Furthermore, baicalin has been detected in the rat cerebrospinal fluid after a single i.v. dose [34], and naringenin and hesperetin have been detected in several rat brain regions after i.v. administration [35,36]. In addition, as also observed with other compounds [37], flavonoid access to the brain tissue can also increase as a result of dysfunction of the blood–brain barrier, a pathological condition of all neurodegenerative diseases [38].

Despite that many studies have shown that several flavonoids display protective activity against oxidative stress-mediated neuronal cell death, recently reviewed in [39], and that many experimental data have pointed out the relevance of mitochondria as a source of intracellular ROS, there is a lack of a systematic study on the modulation of ROS production by brain and heart mitochondria by representative members of the major types of flavonoids. This point is particularly timely, as recently published data have pointed out that within the cells flavonoids can be enriched in mitochondria. Epigallocatechin-3-gallate has been reported to specifically accumulate (90–95%) in the mitochondrial fraction of cerebellar granule neurons in culture, and it has been proposed that due to this the flavonoid afforded protection against mitochondrial oxidative stress-associated insults [40]. Fiorani et al. [41] also measured a considerable accumulation of quercetin in the mitochondria of Jurkat cells exposed to the flavonoid, and suggested that the organelle can represent a reservoir of biologically active quercetin.

It has been reported that high concentrations (50 μM) of some flavonoids inhibit the respiration of rat liver mitochondria [42,43], and more recent works have revealed that quercetin and genistein modulate the activity of the mitochondrial permeability transition pore [44–46]. However, this is a controversial issue as it has also been reported that high concentrations of quercetin (30–50 μM) can also produce stimulation of oxygen consumption by rat liver and kidney mitochondria [45,46]. Noteworthy, it has been shown that the inhibition produced by quercetin and kaempferol is observed only at relatively high and likely supra-physiological levels of these flavonoids, since low micromolar concentrations, up to 10 μM of kaempferol or quercetin, did not inhibit succinate-supported mitochondrial respiration [47]. In this regard it is to be recalled here that previous works have pointed out that quercetin is only a moderate uncoupler of plant mitochondria based on the small effects of this flavonoid on the rate of oxygen consumption [48]. Moreover, Santos et al. have reported a measurable inhibition only for concentrations of quercetin higher than 25 μM [43]. A similar result has been recently reported for inhibition of malate/glutamate-supported respiration of HeLa cells by kaempferol [49]. The effect of 50 μM quercetin on the production of superoxide by mitochondria from different tissues is also a controversial topic, i.e. no effect on rat kidney mitochondria [46] and increase in rat liver mitochondria [45].

Flavonoids have been shown to protect against oxidative stress-mediated neuronal death using cultures of primary neurons and neuronal cell lines [50–52]. The ability of flavonoids to function as ROS scavengers is related to their capability to form stable radicals, a property that is shared by aromatic compounds containing *o*-dihydroxyl groups or close hydroxyl and carbonyl groups. However, the potency of flavonoids as cellular antioxidants cannot be accounted solely in terms of their chemical antioxidant capacity because of the lack of correlation between the reduction potential of flavonoids and their ability to afford protection against oxidative neuronal death induced by different insults using model neuronal cultures [39,51,53,54]. Indeed, studies carried out in different laboratories have shown that flavonoids can afford a large inhibition of cellular ROS production through inhibition of redox enzymes like NAD(P)H oxidases, xanthine oxidase, monooxygenases, ciclooxygenases and lipooxygenases, recently reviewed in [39]. It is to be recalled here that taxifolin (dihydroquercetin) and other flavonoids at relatively high concentrations, i.e. higher than 100 μM of flavonoid, have been shown to inhibit the peroxidase activity of the cytochrome *c*-cardiolipin complex, an activity linked to the very early stages of apoptosis [55].

In this work we have studied the ability of representative members of the major classes of flavonoids to attenuate the rate of hydrogen peroxide production by brain and heart mitochondria. Owing to the potential therapeutic use of the flavonoids which showed a significant inhibition of hydrogen peroxide production by respiring mitochondria, we have also studied their effects on the activity and redox state of major components of the respiratory chain.

2. Materials and methods

2.1. Materials

The flavonoids (−)(−) epicatechin (≥90%), quercetin (>98%), kaempferol (≥96%), apigenin (≥95%) and naringenin (≥95%) were obtained from Sigma-Aldrich (Sigma, St. Louis, MO, USA; Aldrich, Steinhein, Germany). Myricetin (≥95%) was from Fluka. Cyanidin (>96%) and malvidin (>97%) were obtained from Extrasynthese SAS (Lyon, France).

Dimethyl sulfoxide (DMSO), sodium chloride, potassium chloride, magnesium chloride, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, malic acid and potassium cyanide were supplied by Merck (Darmstadt, Germany). Sodium pyruvate was from Boehringer Mannheim, Germany.

All other products were obtained from Sigma, unless specified otherwise.

2.2. Mitochondria and sub-mitochondrial particles

Wistar rats (~300 g) were sacrificed by cervical dislocation and the brain or heart rapidly excised from each animal and immersed in cold mitochondria isolation buffer containing sucrose 0.25 M, EDTA 1 mM and Tris 5 mM (pH 7.4). The brain (without cerebellum) and the heart were weighted and homogenized in a volume (ml) of isolation buffer 3 times the weight (g). The homogenate was centrifuged at 800 *g* for 10 min at 4 °C and the supernatant separated and centrifuged at 12,500 *g* for 10 min at 4 °C. The mitochondrial pellet was resuspended in isolation buffer and always kept on ice during the measurements.

Mitochondrial enzymatic activities were measured with sub-mitochondrial particles prepared by three freeze–thaw cycles to disrupt membranes.

Protein concentration in mitochondrial preparations was estimated using a commercial protein assay kit (Thermo Scientific, Rockford, IL, USA) based in Coomassie blue dye and bovine serum albumin as standard.

2.3. Mitochondrial H_2O_2 production and oxygen consumption

These measurements were carried out with isolated mitochondria in respiration buffer containing sucrose 0.25 M, KH_2PO_4 5 mM, KCl 10 mM, $MgCl_2$ 5 mM and Tris 10 mM (pH 7.4), pyruvate 5 mM, malate 5 mM and ADP 0.2 mM.

Production of H_2O_2 was assayed by the Amplex Red method [56], with 0.1 mg/ml mitochondria in respiration buffer supplemented with horseradish peroxidase 0.2 U/ml and Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) 5 μM. The increase in Amplex Red fluorescence was followed at 37 °C using excitation and emission wavelengths of 530 and 590 nm, respectively.

Oxygen consumption by mitochondria was measured at 37 °C with an Oxygraph Plus DW1 (Hansatech Instruments) electrode. Mitochondria 1 mg/ml in respiration buffer were pre-incubated with the flavonoids 10 min before addition of pyruvate 5 mM and malate 5 mM and recording state 4 respiration rate. State 3 respiration rate was measured in the presence of 0.2 mM ADP.

2.4. Enzymatic activities

SOD activity was measured at 37 °C by the method of auto-oxidation of pyrogallol in 50 mM Tris/HCl, pH 8, with 100 μM pyrogallol, as in [57]. Auto-oxidation of pyrogallol was monitored by the increase of absorbance at 420 nm. One unit of SOD activity is the amount of sample which reduced by 50% the auto-oxidation rate of 100 μM pyrogallol in 2 ml reaction volume, and the calibration of the SOD activity of our mitochondrial preparations is shown in Fig. S1A of Supplementary Data.

Complex I activity of sub-mitochondrial particles was measured as in [58]. The assay buffer consisted of Tris–HCl 10 mM, KCl 50 mM, EDTA 1 mM and KCN 2 mM (pH 7.4). The quinone CoQ_1 was used as electron acceptor in a concentration of 50 μM, except when a different concentration is indicated in the plots. The concentration of mitochondrial protein used was 20 μg/ml. The reaction was initiated by the addition of 75 μM NADH and monitored from the linear decrease of absorbance at 340 nm, at 30 °C. After 2–3 min, rotenone 5 μg/ml was added to evaluate the activity independent of rotenone. With the brain and heart mitochondria preparations used in this work the rotenone-independent activity was always lower than 10% of the total activity measured. Activities in nmoles per min per mg mitochondrial protein were calculated using an extinction coefficient for NADH of 6.23 $mM^{-1}\,cm^{-1}$.

Complex II activity was measured at 30 °C as described in [59]. Sub-mitochondrial particles (5 μg/ml) were suspended in potassium phosphate buffer 50 mM (pH 7.4) supplemented with succinate 20 mM. After 10 min incubation, 2,6-dichlorophenolindophenol (DCPIP) 50 μM, rotenone 2 μg/ml, antimycin A 2 μg/ml and KCN 2 mM were added. The absorbance at 600 nm was recorded for 1 min before addition of CoQ_1 50 μM and, then, the reduction of DCPIP coupled to complex II-catalyzed reduction of CoQ_1 was followed for 3–5 min. Activities were calculated in nmoles of DCPIP reduced per minute per mg of protein, using an extinction coefficient for DCPIP of 19.1 $mM^{-1}\,cm^{-1}$.

The coupled activity of complex II plus III was measured from the rate of ferricyanide reduction in the following medium: sucrose 0.25 M, KH_2PO_4 5 mM, KCl 10 mM, $MgCl_2$ 5 mM and Tris 10 mM (pH 7.4), i.e. the respiration buffer without substrates described above, supplemented with succinate 10 mM, potassium ferricyanide 0.8 mM and KCN 0.5 mM. After thermal equilibration at 30 °C, the reaction was initiated with 100 μg/ml mitochondrial protein and the change in absorbance at 420 nm was monitored. Activity was calculated in nmoles of ferricyanide reduced (extinction coefficient of 1 $mM^{-1}\,cm^{-1}$) per minute per mg of protein.

2.5. Spectroscopic studies: cytochrome c reduction and difference spectra

The changes in the absorption spectra of cytochrome *c* induced by flavonoids were studied with solutions of oxidized cytochrome *c* (from horse heart) at a concentration of 5 μM in mitochondria isolation buffer without substrates, see above. Absorption spectra of the solutions were recorded at 37 °C before and at the indicated time intervals until 30 min after the addition of the flavonoid. Absorption spectra of the flavonoids in the same buffer were also recorded as experimental blanks, and found to be negligible above 430 nm.

The kinetics and equilibrium of the reaction between flavonoids and cytochrome *c* were studied with solutions of cytochrome *c* 20 μM in 10 mM potassium phosphate buffer (pH 7.4). Cytochrome *c* reduction was followed at 550 nm at 37 °C. Complete reduction of cytochrome *c* was achieved by addition of an excess of dithionite (sodium hydrosulfite; Aldrich, Steinhein, Germany).

Absorption difference spectra of mitochondria incubated with flavonoids were obtained with 2 nm resolution using a Hewlett–Packard model 8451A diode array spectrophotometer. The diode array spectrophotometer allows to measure simultaneously the absorbance in the range of wavelengths selected, making possible to detect spectral changes associated with coupled redox pairs that show separate absorption bands. Mitochondria were suspended at a concentration of 0.75 mg/ml in 1 ml of mitochondria isolation buffer (described above). The suspension was kept under mild stirring for 10 min at 25 °C and the absorption spectrum in the range 400–700 nm was acquired. In separate assays, it was observed that no additional changes occurred until 20 min incubation. Then, the flavonoid was added at a final concentration of 20 μM and, after 10 min incubation, the absorption spectrum of the suspension of mitochondria + flavonoid was recorded, subtracting the mitochondria spectrum previously obtained and stored in the diode array spectrophotometer memory. Absorption spectra of 20 μM flavonoid solutions in the same buffer were also recorded and subsequently subtracted from the mitochondria spectra to obtain the final difference spectra.

3. Results

3.1. Effects of flavonoids on mitochondrial hydrogen peroxide production and oxygen consumption

It is well established that the mitochondrial electron transport chain produces superoxide ($O_2^{\cdot-}$) that is rapidly converted to hydrogen peroxide (H_2O_2) by the action of mitochondrial superoxide dismutase, see e.g. [60,61].

We have isolated mitochondria from rat brain and heart and measured the rate of release of H_2O_2 using the Amplex Red assay. This fluorescent probe is highly sensitive for H_2O_2 [56] and has been widely used as a reliable, sensitive and robust method to measure H_2O_2 generation by mitochondria [9,60,62,63].

Measurements were made with mitochondria suspended in a sucrose-based respiration buffer containing pyruvate, malate and ADP, as described in detail in Materials and methods. In these conditions, we observed a slightly higher production rate of H_2O_2 by heart compared to brain mitochondria, 0.26 ± 0.02 vs 0.18 ± 0.04 nmol H_2O_2/min/mg of mitochondrial protein, respectively.

A group of eight flavonoids representative of the major classes of these compounds was initially selected for this study and their effects on mitochondrial H_2O_2 production was assessed. The group included flavanols (epicatechin), flavonols (quercetin, kaempferol, myricetin), flavones (apigenin), flavanones (naringenin) and anthocyanidins (cyanidin and malvidin). The screening of the response of Amplex Red/Peroxidase detection system used for H_2O_2 measurements pointed out that the flavonoids myricetin, cyanidin and malvidin should be discarded in these studies, because of their direct interference with the Amplex Red/Peroxidase detection method (Fig. S2A of Supplementary Data). However, the increase of the fluorescence of the Amplex Red/Peroxidase detection system elicited by epicatechin was canceled by supplementation of the media with 2 mM EGTA (Fig. S2A of Supplementary Data). Thus, the effect of epicatechin on mitochondrial H_2O_2 production was assayed in the presence of EGTA. The good response of the Amplex Red/Peroxidase detection system in the presence of 10 μM of the flavonoids apigenin, epicatechin, kaempferol, naringenin,

and quercetin was further assessed by titration of the fluorescence increase after addition of different amounts of H_2O_2. As shown in the Fig. S2B of Supplementary Data, the response of the detection system up to 100–200 nM H_2O_2 was not significantly affected by the flavonoids kaempferol, quercetin, apigenin, and naringenin under the routine conditions used in this work. The small effect of the flavonoid epicatechin in presence of EGTA, i.e. a decrease of approx. 10% of the increase of fluorescence, was taken into account in the calculations of mitochondrial H_2O_2 production. Although it has been reported that flavonoids are inhibitors of the horseradish peroxidase activity displaying IC_{50} values in the micromolar concentration range [64], our results pointed out that the H_2O_2 assay is robust enough to function properly most likely because of the excess of horseradish peroxidase activity used in the assay medium.

As shown in Fig. 1, the flavonoids showed different effects on the rate of H_2O_2 production by mitochondria. Moreover, there were no indications of transient H_2O_2 direct-scavenging by flavonoids, i.e. no significant changes of the fluorescence were observed immediately after the addition of flavonoids. This point was further assessed with blank experiments (without mitochondria). It is to be noted that the addition of up to 10 μM flavonoid did not elicit a significant change of the pH, measured to be lower than 0.02 pH units. As also shown in Fig. 1, there is some variation between the effects of different flavonoids on H_2O_2 production rate by brain and heart mitochondria. Quercetin, kaempferol and epicatechin effectively decreased mitochondrial H_2O_2 production rate, and kaempferol at a concentration of 10 μM was able to completely block H_2O_2 release by both brain and heart mitochondria. Apigenin and naringenin at concentrations of 1 and 10 μM showed no effects on H_2O_2 production rate by mitochondria from brain and heart.

On these grounds, we decided to study in more detail the molecular basis for the inhibition of H_2O_2 production by mitochondria afforded by quercerin and kaempferol. In addition, taking into account that epicatechin and apigenin have been described to have very potent cytoprotective actions and that they are also representatives of other major flavonoid classes, we decided to examine in more detail the interaction of these 4 compounds with mitochondria.

The possibility that the decrease of mitochondrial H_2O_2 production observed in presence of these flavonoids could be due to inhibition of mitochondrial SOD activity was assessed by measuring this activity using pyrogallol. Our results pointed out that at 10 μM the flavonoids that produce a large inhibition of mitochondrial H_2O_2 release, i.e. kaempferol, quercetin and epicatechin, afford at most only 10% inhibition of mitochondrial SOD activity (see Fig. S1B of Supplementary Data). This result is in good agreement with the lack of inhibition of mitochondrial SOD by diphenolic compounds, like catechol and noradrenaline, and other plant phenols like coumaric acid, ferulic acid and gentisic acid, reported in [65].

In the screening of flavonoids presented above, quercetin and kaempferol revealed remarkable abilities to inhibit mitochondrial H_2O_2 production. The inhibition curves in Fig. 1D show that these flavonoids at concentration of 5 μM almost completely abolished H_2O_2 production by brain mitochondria. These results yielded

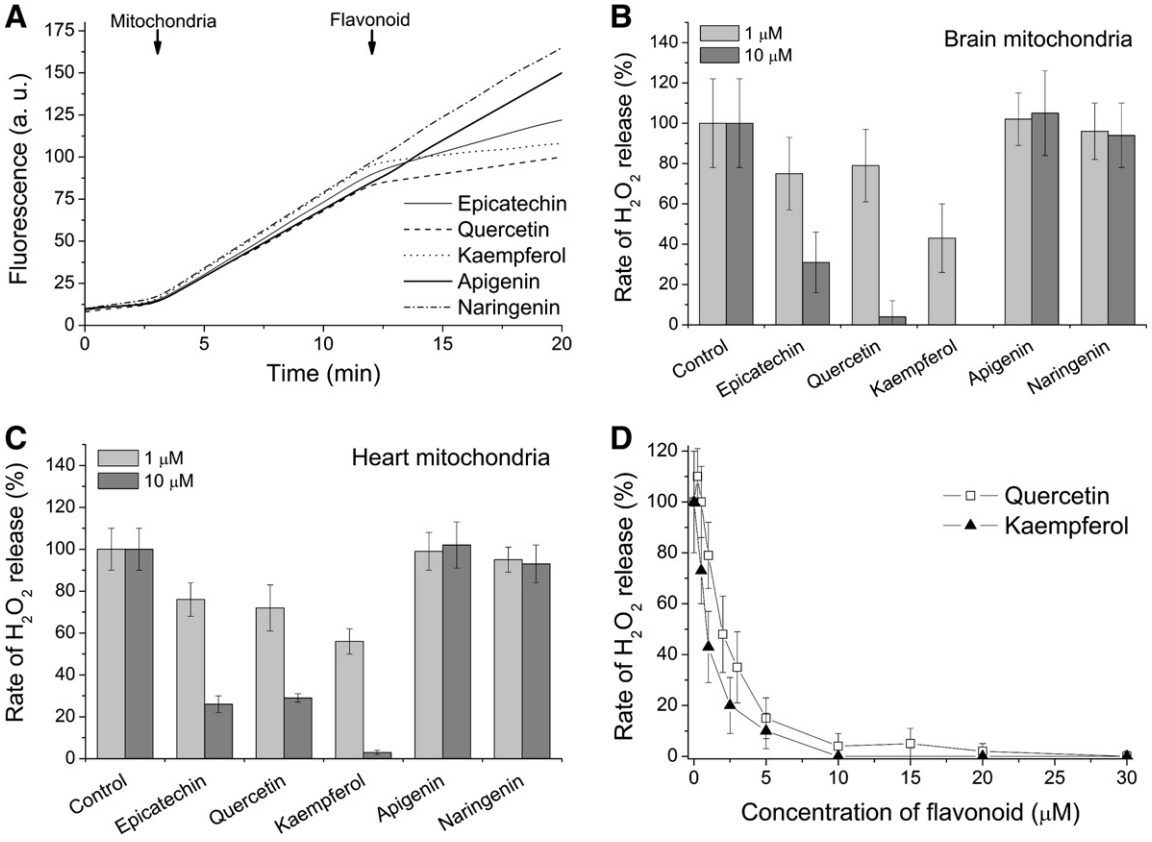

Fig. 1. Effects of flavonoids on mitochondrial production of H_2O_2. (A) Representative experimental traces of oxidation of Amplex Red by mitochondria and the effect of flavonoid addition. Production of H_2O_2 by mitochondria (0.1 mg/ml) in respiration buffer was detected by the Amplex Red method as described in the Materials and methods section. The experimental traces shown were obtained with rat brain mitochondria and with addition of 10 μM flavonoid concentration, although it is to be noted that similar traces were obtained with rat heart mitochondria. Panels B and C, effects of 1 and 10 μM concentration of flavonoids on the H_2O_2 production rate of mitochondria isolated from rat brain and heart, respectively. (D) Effect of quercetin and kaempferol concentration on H_2O_2 production by rat brain mitochondria. The results are expressed as percentage of H_2O_2 production rate relative to the rate observed in the absence of flavonoid, which was taken as control (100% value). The 100% values of H_2O_2 production rate (in nmol H_2O_2/min/mg) in the controls were 0.18 ± 0.04 (brain) and 0.26 ± 0.02 (heart). The data represented is the mean ± standard error from measurements made with at least three preparations of each type of mitochondria.

values of 50% inhibitory concentration (IC_{50}) for quercetin and kaempferol of 1.8 and 0.9 μM, respectively.

The effect of these flavonoids on mitochondrial oxygen consumption was also assessed. Epicatechin, quercetin, kaempferol and apigenin up to concentrations of 10 μM showed no significant effects on oxygen consumption rate by mitochondria, neither in state 4 nor in state 3 (Fig. S3 of Supplementary Data). Apigenin was also included as reference flavonoids, since it did not afford a significant inhibition of the rate of hydrogen peroxide production by respiring mitochondria. Thus, this result excluded a direct relationship between inhibition of H_2O_2 production by these flavonoids and oxygen consumption by mitochondria.

3.2. Interaction of flavonoids with respiratory chain components

The inhibitors of the respiratory chain rotenone and antimycin A increased H_2O_2 production by mitochondria isolated from rat heart and brain (Fig. 2A), in agreement with the results reported in other studies [9,60,62].

The ability of quercetin and kaempferol to prevent mitochondrial H_2O_2 production was evaluated with brain mitochondria in presence of these inhibitors and, as shown in Fig. 2 (B and C), the potency of the flavonoids was very similar to that observed against basal H_2O_2 production of untreated mitochondria.

It is widely accepted that the mitochondrial inhibitors used in this study amplify mitochondrial H_2O_2 production by blocking particular steps of the respiratory chain and increasing electron leakage to oxygen. The potent capacity of quercetin and kaempferol to counteract H_2O_2 production stimulated by the respiratory chain inhibitors suggested that these flavonoids are likely modulating the electron flow through some component of the electron transport chain.

3.3. Modulation of complex I by flavonoids

Complex I of the electron transport chain has been reported to be the main source of superoxide production by mitochondria [9,60,62]. The effect of epicatechin, quercetin, kaempferol and apigenin on complex I activity of brain sub-mitochondrial particles was studied and the results are presented in Fig. 3. Complex I activity was measured as NADH:CoQ_1 oxidoreductase activity and it was found that addition of quercetin, kaempferol and apigenin inhibited enzymatic activity (Fig. 3A and B). These flavonoids at a concentration of 10 μM produced approximately 30% inhibition under these experimental conditions (Fig. 3B). In contrast, 10 μM epicatechin showed a very weak effect on complex I activity, approximately 10% inhibition. Despite that there were no indications of an initial lag phase in the inhibition produced by these flavonoids, in separate experiments, sub-mitochondrial particles were pre-incubated for 30 min (at 30 °C) with the flavonoids before assaying the enzymatic activity, and the results obtained were identical to those shown in Fig. 3B obtained without pre-incubation with the flavonoid. At higher concentrations, kaempferol exhibited a more potent inhibitory capacity than quercetin (Fig. 3C). Apigenin at concentrations up to 10 μM showed an inhibitory potency similar to kaempferol, but it was not possible to evaluate the effect of higher concentrations since apigenin caused precipitation of mitochondria in the assay medium.

As flavonoids have chemical homology with the quinone moiety of CoQ, the possibility that flavonoid inhibition could be due to competition for binding to the quinone binding sites of complex I deserved to be experimentally tested. The results shown in Fig. 3 were obtained using 50 μM CoQ_1 as electron acceptor for complex I. In a first set of competition experiments, sub-mitochondrial particles were pre-incubated with CoQ_{10} before assay of complex I activity with 20 μM CoQ_1, and this led to attenuation of the inhibition by quercetin and kaempferol (Fig. 4A). Noteworthy, CoQ_{10} protected more efficiently against the inhibition afforded by quercetin than against the inhibition produced by kaempferol. It is to be noted that no oxidation of NADH occurred when CoQ_{10} was supplied to the assay medium as the only electron acceptor, confirming that it is not used as Complex

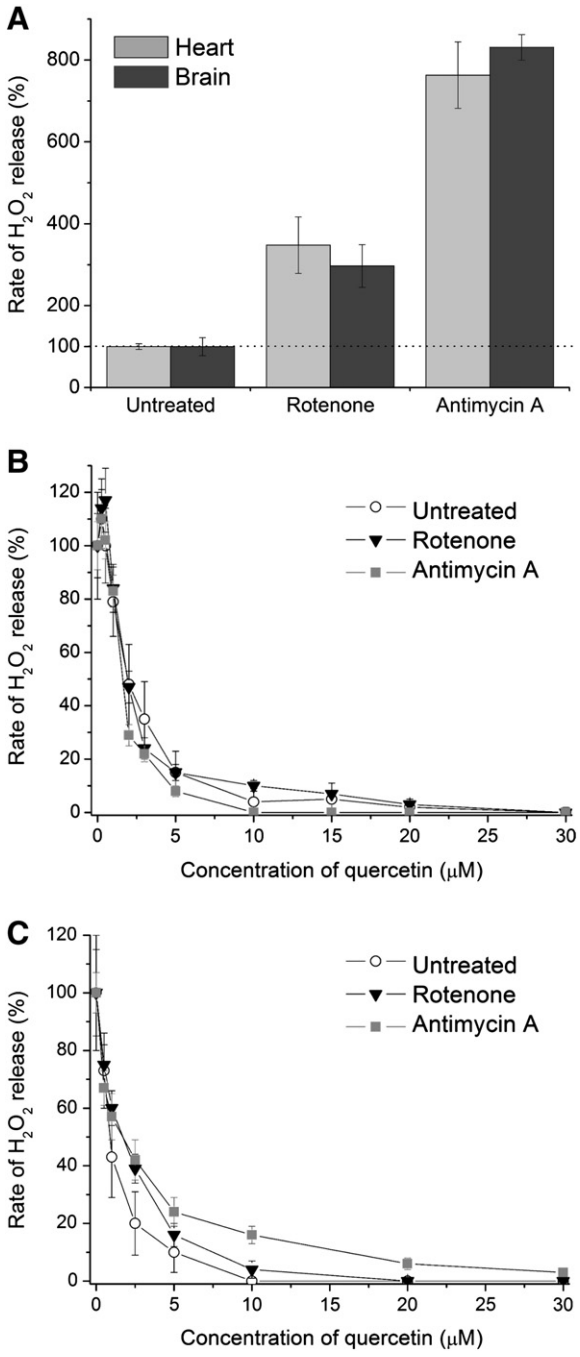

Fig. 2. Effects of mitochondrial inhibitors on H_2O_2 production by mitochondria isolated from rat heart and brain (A). Production of H_2O_2 was measured as in Fig. 1. Rotenone, and antimycin A were used in concentrations 10 μM and 2 μM, respectively. Results are shown as percentage of H_2O_2 production rate relative to the production rate observed with the same mitochondria in the absence of the inhibitors (untreated). The values of H_2O_2 production rate of untreated mitochondria (in nmol/min/mg) were 0.26 ± 0.02 (heart) and 0.18 ± 0.04 (brain). Panels B and C represent the capacity of quercetin and kaempferol to inhibit H_2O_2 production by brain mitochondria stimulated with mitochondrial inhibitors. Results are shown as percentage of production rate relative to the rate observed in the absence of flavonoid, which was normalized to 100%. The 100% values of production rate (in nmol H_2O_2/min/mg) were 0.18 ± 0.04 (untreated), 0.53 ± 0.09 (rotenone) and 1.50 ± 0.07 (antimycin A). All the results in panels A to C are the mean ± standard error from measurements made with at least three preparations of each type of mitochondria.

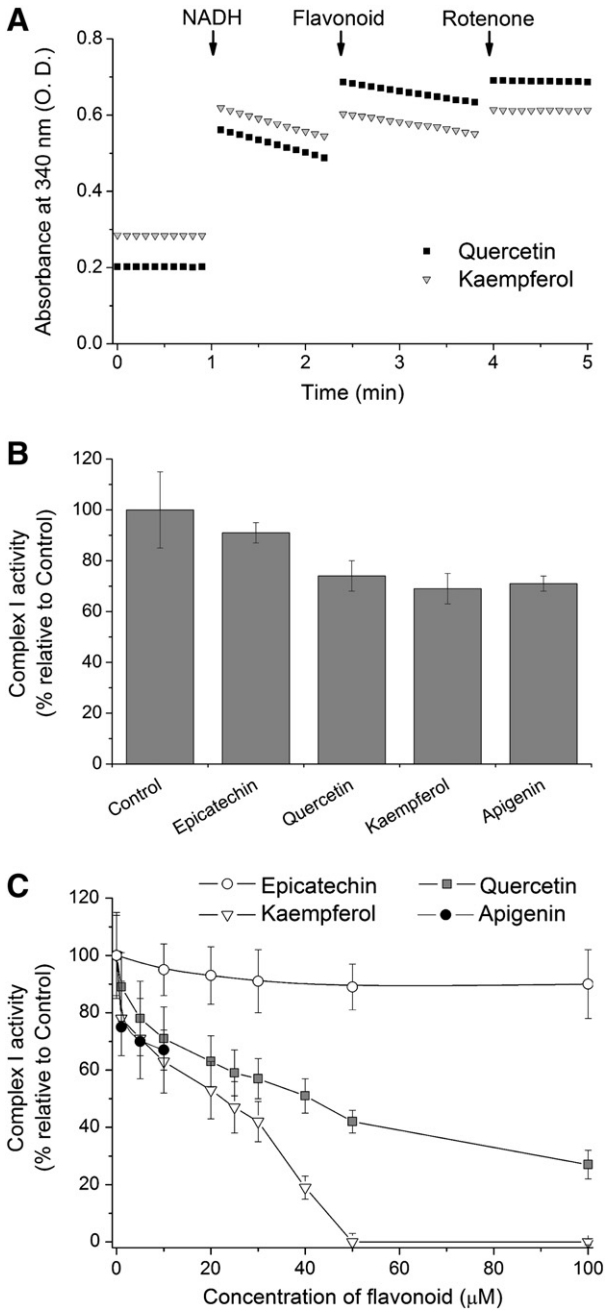

Fig. 3. Effects of flavonoids on complex I activity of rat brain sub-mitochondrial particles. (A) Representative experimental traces of complex I activity and the effect of flavonoid addition. Experiments in this figure were done at 30 °C with mitochondria (20 μg protein/ml) in the following medium: Tris–HCl 10 mM, KCl 50 mM, EDTA 1 mM, KCN 2 mM and CoQ_1 50 μM (pH 7.4). After thermal equilibration of the cuvette containing mitochondria in the buffer, the reaction was started with NADH and, after approximately 1 min, the flavonoid was added (20 μM in the experiments shown in this figure). The rapid increase of absorbance observed when the flavonoids were added is due to the absorption at 340 nm of quercetin and kaempferol. At the end of the assays, rotenone was added to determine the putative contribution of complex I-independent NADH oxidase activity, which was lower than 10% of total activity. (B) Inhibition of complex I activity by flavonoids at a concentration of 10 μM. (C) Titration of the NADH oxidase activity of complex I with epicatechin, quercetin, kaempferol and apigenin. The effect of apigenin concentrations higher than 10 μM was not studied, because above 10 μM apigenin induced mitochondria precipitation in the assay medium. Inhibition results are presented as percentage of activity relative to the activity of the same mitochondrial preparation in the absence of flavonoid and presence of the volume of solvent (DMSO) added with the flavonoid. The absolute value of the 100% specific activity was 179 ± 27 nmol NADH oxidized/min/mg. The results shown are the mean ± standard error of measurements made with 3 to 6 preparations of mitochondria.

I substrate when sub-mitochondrial particles are assayed (*results not shown*, see also [58]). On these grounds, we decided to measure the inhibitory potency of flavonoids varying the concentration of CoQ_1 in the assay medium (Fig. 4B). These results showed that the capacity of quercetin, kaempferol and apigenin to inhibit complex I decreased as CoQ_1 concentration increased.

3.4. Modulation of other components of the respiratory chain

Complexes II and III also use CoQ_{10} as substrates and the effect of the selected flavonoids on the activity of these complexes was investigated. As illustrated in Fig. S4 of Supplementary Data, epicatechin, quercetin, kaempferol and apigenin up to concentrations of 10 μM did not show any significant modulation of complex II activity, but elicited a weak stimulation of the rate of succinate-driven ferricyanide reduction produced by mitochondria, i.e. approx. 25% stimulation for quercetin and kaempferol. However, the complex III specific activity in the presence of 10 μM of these flavonoids, calculated from the inhibition afforded by 2 μM antimycin A, was not significantly different from the result obtained for the control measured in the absence of flavonoid, namely 150 ± 15 nmoles/min/mg mitochondrial protein.

The assays of the activity of complexes III and IV involve the use of cytochrome *c* as substrate [59]. When performing these assays we noticed that some of the flavonoids altered the reduction state of the cytochrome *c*, and this effect could account for the apparent

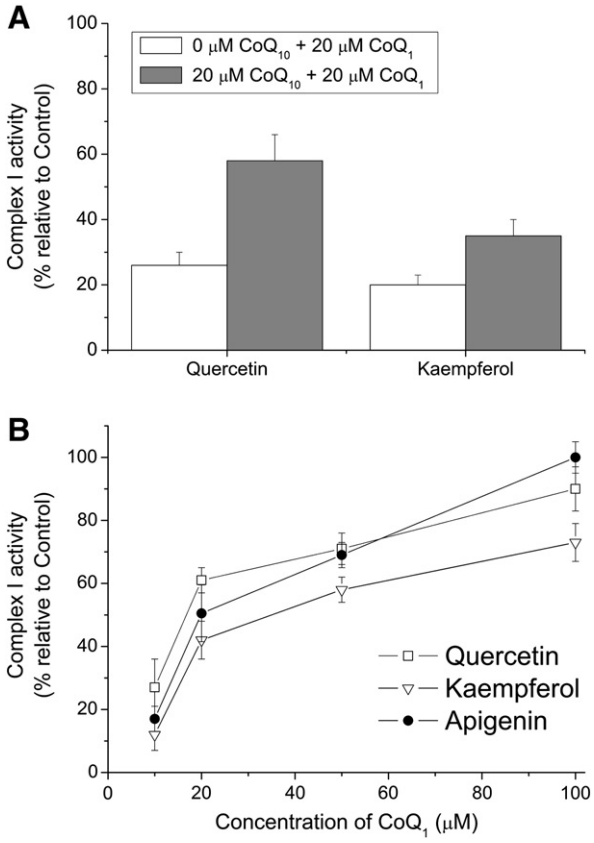

Fig. 4. The inhibition of complex I activity of rat brain sub-mitochondrial particles by flavonoids is antagonized by CoQ_{10} (A) and CoQ_1 (B). Flavonoids (10 μM) were directly added to the assay medium of complex I activity in different conditions. In panel A, activity was measured in presence of 20 μM CoQ_1 after incubation of sub-mitochondrial particles for 20 min at 30 °C with 20 μM CoQ_{10} in DMSO or with only DMSO (without CoQ_{10} addition). In panel B, complex I activity was measured with different concentrations of CoQ_1 in assay medium (without any addition of exogenous CoQ_{10}). The results shown are the mean ± standard error from measurements made with three preparations of mitochondria.

weak stimulation of complex II plus III coupled activity measured from the reduction of ferricyanide.

Epicatechin, quercetin and kaempferol readily reduced cytochrome *c* (Fig. 5). The results obtained with apigenin are not included in this figure, because apigenin did not elicit detectable spectral changes in the solution of cytochrome *c*. Reduction of the cytochrome *c* was established by the spectral changes known to monitor the reduction of cytochrome *c* by standard reducing agents like dithionite (sodium hydrosulfite). Addition of the flavonoids to a cytochrome *c* solution caused the progressive appearance of distinct absorption bands at 550 and 520 nm, and the shift of the 410 nm band to slightly larger wavelengths in the absorption spectrum of the solution, as shown in Fig. 5A for kaempferol. As illustrated with epicatechin in Fig. 5B, the kinetics of reaction between the flavonoids and the cytochrome was concentration dependent.

The study of the stoichiometry of the reduction of cytochrome *c* by epicatechin, quercetin and kaempferol pointed to a 2:1 relation between cytochrome *c* and flavonoid (Fig. 5C). Thus, each flavonoid molecule is able to transfer 2 electrons, reducing 2 cytochrome *c* molecules. A small fraction of oxidized cytochrome *c* still remained even in the presence of an excess of the flavonoids (Fig. 5C). As oxygen removal by gassing the buffer with N_2 and later measurements in sealed cuvettes led to the same result re-oxidation of cytochrome *c* by the oxygen dissolved in the solution seemed unlikely. Therefore, these results lend support to the hypothesis that this is the reduction level attained at the equilibrium of this redox reaction, and applying the Nernst equation this will be consistent with a $\Delta E^{0'}$ of 0.046 ± 0.005 V.

The possibility of interaction of flavonoids with complex IV in mitochondria was explored by spectroscopic methods. Reduction of complex IV produces a significant increase of absorption at 600 nm [66]. Difference spectra of mitochondria incubated with flavonoid minus mitochondria pointed out that none of the four flavonoids tested reduced complex IV. Respiring mitochondria incubated with epicatechin and apigenin did not show well resolved bands in this range (*data not shown*).

4. Discussion

The results of this study have pointed out that different flavonoids can produce distinct effects on the rate of production of H_2O_2 by respiring mitochondria. Up to 10 μM concentration of flavonoid quercetin, kaempferol and epicatechin were found to elicit a significant inhibition, whereas 2 flavonoids (apigenin and naringenin) had no statistically significant effect on the rate of H_2O_2 production by respiring brain and heart mitochondria. Among the flavonoids studied in this work, kaempferol and quercetin were the most potent as inhibitors of the rate of H_2O_2 production by brain mitochondria leading to nearly 100% inhibition with 10 μM flavonoid, and showed IC_{50} values of 0.9 and 1.8 μM, respectively. Also, this inhibition cannot be simply due to a direct chemical reduction of produced H_2O_2 for two major reasons: (i) these concentrations of flavonoid did not produce a significant decrease of the Amplex Red fluorescence signal when added before this reagent to respiration buffer supplemented with nanomolar H_2O_2 concentrations, and (ii) the inhibitory effect of the flavonoid was steady and lasted for at least 30 min. However, this inhibition is not due to a significant slowdown of the oxygen consumption rate in respiring mitochondria, as up to 10 μM kaempferol and quercetin did not significantly affect the oxygen consumption rate. These results are consistent with previous reports showing lack of inhibition of succinate-driven oxygen consumption by mitochondria in the presence of 10 μM kaempferol or quercetin [47], as well as with the lack of effect of other flavonoids (taxifolin, catechin and galangin) on mitochondrial respiration using both glutamate/malate and succinate as substrates [42].

Mitochondrial respiratory complexes I and III have been reported to be the major systems involved in ROS production by respiring mitochondria (reviewed in [2,9]). As micromolar concentrations of

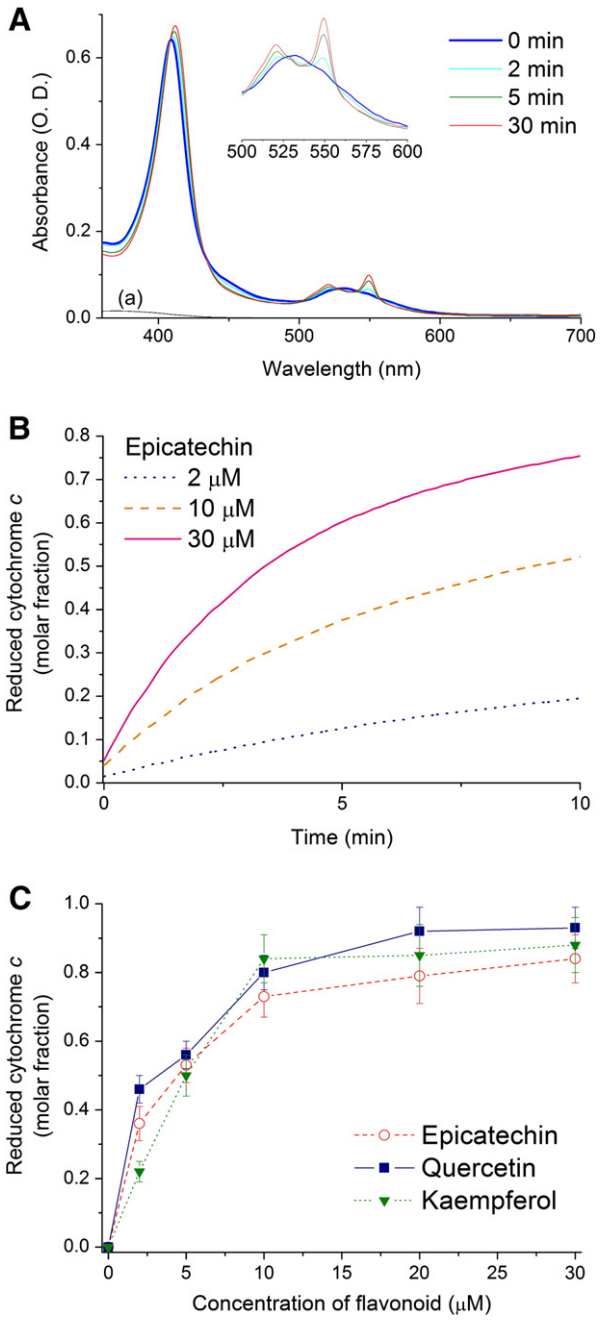

Fig. 5. Reduction of cytochrome *c* by quercetin, kaempferol and epicatechin. Panel A shows the changes of the absorption spectrum of cytochrome *c* elicited by kaempferol. The flavonoid was added at a final concentration of 1 μM to a 5 μM oxidized cytochrome *c* solution and the reaction was followed for 30 min. The absorption spectra of 1 μM solution of kaempferol is also presented (spectra a in panel A). Inset: detail of the spectral changes in the 500–600 nm range. Similar spectral changes were observed with quercetin and epicatechin (*data not shown*). Panel B shows the kinetics of reduction of cytochrome *c* (20 μM) by epicatechin at different concentrations, followed at 550 nm. Complete reduction of cytochrome *c* was achieved with the addition of an excess of dithionite, a well-known reductant of cytochrome *c*. All these reactions were performed in 10 mM potassium phosphate buffer (pH 7.4), at 37 °C. The data shown are representative of the results obtained in triplicate experiments. Panel C shows the dependence upon the concentration of flavonoids of the extent of reduction of cytochrome *c*. Flavonoids were added to 20 μM oxidized cytochrome *c* in 10 mM potassium phosphate buffer (pH 7.4). Reduction of cytochrome *c* was monitored at 550 nm until equilibrium was achieved at 37 °C. Complete reduction of cytochrome *c* was obtained with the addition of an excess of dithionite. Results are the mean ± standard error from triplicate measurements.

several flavonoids have been demonstrated to inhibit different ROS-producing enzymes, such as lipoxygenases [67], xanthine oxidase [68] and NADH and NADPH oxidases [52,69], we have experimentally assessed that the inhibition afforded by kaempferol and quercetin on the rate of H_2O_2 production by rat brain mitochondria was due to impairment of ROS production by the main complexes of the respiratory chain.

In the presence of the mitochondrial inhibitors rotenone and antimycin A, production of H_2O_2 was stimulated, in accordance with other studies [9,70]. Basal production of ROS by mitochondria in normal conditions can have several sources as indicated above, however, the increased production of O_2^- in the presence of rotenone is known to come from complex I, while antimycin A exacerbates ROS generation by complexes I and/or III [9,60,62]. Quercetin and kaempferol showed similar potencies as inhibitors of H_2O_2 production by mitochondria in basal and inhibitor-stimulated conditions, which suggested that they inhibit complex I, and possibly also complex III, ability to generate O_2^-. Indeed, we found that quercetin, kaempferol and apigenin inhibit complex I (rotenone-sensitive NADH:CoQ_1 oxidoreductase) activity in brain sub-mitochondrial particles. However, our results excluded the inhibition of the activity of complex II or of the coupled activity of complexes II and III, leaving complex I as the major target of quercetin and kaempferol for the inhibition of mitochondrial ROS production.

With respect to quercetin and kaempferol, it is to be noted that this conclusion is in good agreement with the results by Silva et al. [47] who found that these compounds have no significant effects on succinate-supported respiration rate of brain mitochondria. This conclusion is also consistent with recent results reported by Filomeni et al. [49], who had shown that pre-treatment of HeLa cells with 200 μM kaempferol inhibited malate/glutamate-supported respiration, whereas no significant effect was observed in succinate-supported respiration, therefore suggesting that this flavonoid affected mitochondrial electron transfer chain at the level of complex I. Also kaempferol has been reported to strongly attenuate rotenone-induced oxidation of mitochondrial proteins in neuroblastoma cells [71].

The titration of the NADH:CoQ_1 oxidoreductase activity of Complex I of rat brain mitochondria shows that 50 μM quercetin and kaempferol produced more than 50% inhibition, while below 10 μM they afford only a weak inhibition, i.e. less than 20% of this activity. Noteworthy, we found that the inhibition of complex I activity was dependent on the concentration of CoQ present in the medium, suggesting that the flavonoids affect binding of the coenzyme substrate to the complex. Complex I is known to have two distinct sites for quinones reduction: the physiological, proton-translocating, inhibitor (rotenone and piericidin A)-sensitive site, known as "hydrophobic site"; and a non-energy-transducing, inhibitor-insensitive site, the "hydrophilic site". King et al. [72] presented strong evidence that the "hydrophilic site" is located at the flavin active site for NADH oxidation. Recent data from different studies suggest that the "hydrophobic site" is located in a quinone (and inhibitor) binding pocket in a cavity between the PSST and the 49-kDa subunits, next to iron–sulfur cluster N2 in the complex, as reviewed in [73]. Under the conditions of our experiments, the flow of electrons from NADH oxidation is channeled through Complex I to the quinone in the "hydrophobic site", as shown by the almost complete (>90%) inhibition of the activity by rotenone. Therefore, it is not probable that the inhibitory effect of flavonoids on NADH:CoQ_1 oxidoreductase activity occurs at the "hydrophilic" quinone reduction site.

Hydrophobic inhibitors bind to different but partially overlapping binding sites within a large common binding domain in the above cited quinone and inhibitor binding pocket, as it has been pointed out by diverse and complementary experimental approaches [73–75]. On these grounds, the differences in the extent of inhibition of the NADH:CoQ_1 oxidoreductase activity of Complex I produced by the flavonoids studied in this work (epicatechin, kaempferol, quercetin and apigenin), as well as the differences on the efficiency of protection by CoQ_{10} and CoQ_1 against this inhibition, may arise from different affinities of these flavonoids to these partially overlapping sites or from different local conformational effects in the inhibitor binding pocket of Complex I. The site(s) of reduction of oxygen to produce superoxide anion in complex I remain unknown, but the more probable candidates proposed are the ubisemiquinone species, the flavin and iron–sulfur cluster N2 [76,77]. Thus, our results pointing out kinetic competition between coenzyme Q_1 and the flavonoids kaempferol and quercetin strongly suggest that these flavonoids bind to complex I in the quinone and inhibitor binding pocket as it seems to happen with rotenone, despite that these flavonoids and rotenone showed opposed effects in H_2O_2 production by mitochondria. In this regard, it is worth noting that among the different inhibitors of complex I, Fato et al. [77] found that some inhibitors induced an increase in the production of ROS while others prevented the same activity. These authors explained these observations on the basis of a two-step mechanism of electron transfer to ubiquinone at the active site, involving two distinct conformations differently blocked by the inhibitors, being one of the conformations competent for electron delivery to oxygen but not the other. In this sense, two modes of action were also previously reported for different inhibitors of complex I, all of which affected the electron transfer step from the high potential iron-sulfur cluster to ubiquinone [78]. It is to be recalled that pulse radiolysis studies yielded one-electron reduction potentials close to 0.4 V for semiquinone radicals [79], a value that is close to the reduction potential of flavonoids kaempferol, quercetin and epicatechin at pH 7.4, which lie between 0.3 and 0.4 V [80]. It is to be noted that total H_2O_2 produced during our assays is well below the IC_{50} values obtained in this work for these flavonoids, i.e. a nearly steady inhibition should be expected even if they act as sacrificial antioxidants. Indeed, the rate of H_2O_2 production by our respiring mitochondria preparations is close to 0.2 nmoles H_2O_2/min/mg mitochondrial protein, and this implies that during the average duration of our activity assays done with 0.1 mg mitochondrial protein/ml in the presence of the flavonoid (15 min) the total production of H_2O_2 is ca. 0.3 nmoles/ml = 0.3 μM H_2O_2. Thus, *in situ* kinetic competition between the reactions of reduction of flavonoids and oxygen or alteration of the conformation of complex I competent for electron delivery to oxygen due to the binding of the flavonoid in the hydrophobic pocket could provide the simplest hypothesis to account for the inhibition by flavonoids of electron leakiness through the semiubiquinone radical. A better three-dimensional resolution of complex I conformations in the absence and presence of flavonoids, and also of their binding site(s) is needed to critically discriminate between alternate possible molecular mechanisms for inhibition of the NADH:CoQ_1 oxidoreductase activity and superoxide anion production by the flavonoids.

There is a growing consensus that complex I is the major source of O_2^- within mitochondria *in vivo* [9]. Complex I has been proposed to be the initial site of ischemia-elicited damage to mitochondria in the heart and the likely source of ROS in models of heart failure [1], and deficiencies in complex I function are well documented in neurodegenerative diseases [2]. Therefore, the inhibition of complex I activity by concentrations of quercetin, kaempferol and apigenin in the low micromolar range and, in accordance, the potent inhibition by quercetin and kaempferol of H_2O_2 release by mitochondria may have a high relevance in the context of prevention of neurodegeneration and cardiovascular disease progression. Our results also point out that coenzyme Q deficient mitochondria should be more prone to inhibition of complex I activity by these flavonoids, due to the observed competition between coenzyme Q and the flavonoid. Despite that flavonoids can be more concentrated in mitochondria than in other subcellular compartments [40,41], the concentrations of flavonoids required to elicit a large inhibition of mitochondrial complex I activity, i.e. above 10 μM, are more than 10-fold higher than those

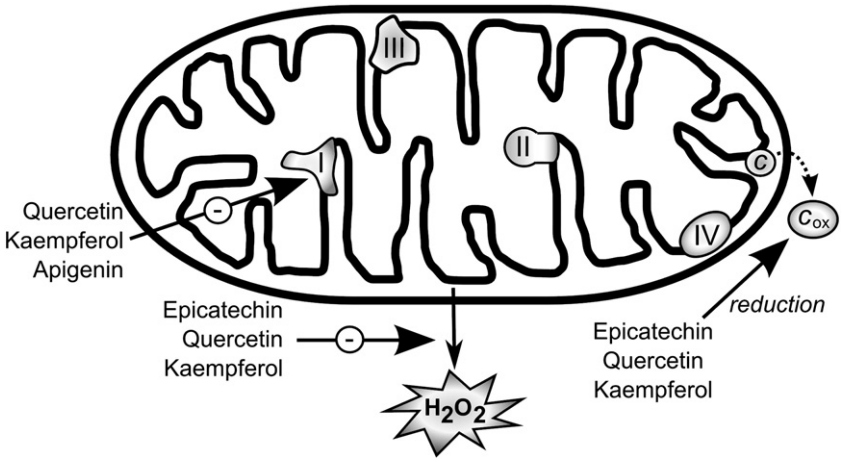

Fig. 6. Effects of flavonoids on mitochondrial production of hydrogen peroxide and modulation of respiratory chain components. (I): complex I; (II): complex II; (III): complex III; (IV): complex IV; (c): cytochrome *c*; (c_{ox}): oxidized cytochrome *c*; (−): inhibition.

found in the brain after controlled dietary intake, see [39]. Moreover, epicatechin up to 100 μM only produced a weak inhibition of the NADH:CoQ_1 oxidoreductase activity of Complex I, less than 20% inhibition, an inhibition which is much lower than the extent of observed inhibition of the mitochondrial H_2O_2 production by this flavonoid. It is to be noted, however, that the rate of hydrogen peroxide production by our respiring mitochondria preparations is 0.5–1% of their rate of oxygen consumption, ca. 0.18–0.26 nmoles H_2O_2/min/mg mitochondrial protein versus nearly 50 ng atom O/min/mg mitochondrial protein using pyruvate + malate as respiratory substrates.

In addition, our results have unraveled that one molecule of epicatechin, kaempferol and quercetin can afford the reduction of two molecules of purified cytochrome *c* at physiological pH, but not of complex IV. The fact that half-reduction of 20 μM cytochrome *c* takes place with concentrations of flavonoid close to 5 μM points out that this reaction has a high equilibrium constant value, and this implies that it should be efficiently taking place at the low concentrations of flavonoids that can be attained in the brain after feeding or by intraperitoneal or intravenous injections of moderate doses of flavonoids, reviewed in the reference [39]. As the reported half-reduction potentials of these flavonoids in aqueous solution are higher than the half-reduction potential of cytochrome *c* [80–82], these results point out that binding of epicatechin, kaempferol and quercetin to cytochrome *c* elicits either a large decrease of the half-reduction potential of the flavonoids or a significant conformational change of cytochrome *c* that increases the heme reduction potential or both. The failure of apigenin to reduce cytochrome *c* is consistent with this hypothesis, as the semireduction potential of apigenin in water, 0.86 V [83], is higher than those reported for epicatechin, kaempferol and quercetin, ranging from 0.29 V to 0.43 V [80,82]. Owing to the key role of oxidized cytochrome *c* in the early stages of cellular apoptosis, reviewed in [84], this effect of flavonoids is likely to contribute to the observed protection of flavonoids against the apoptosis-mediated cell death [40,50]. Further work is required to assess the extent of flavonoid-induced reduction of cytochrome *c* bound to mitochondria, as likely lipid-binding would interfere with the interaction of other lipophilic molecules such as flavonoids with cytochrome *c*.

4.1. Conclusions

The schematic diagram of Fig. 6 summarizes the major findings reported in this work. Briefly, different flavonoids have distinct effects on H_2O_2 production by brain and heart mitochondria, being quercetin and kaempferol the most potent as inhibitors of H_2O_2 production with IC_{50} values in the low micromolar range. For these flavonoids the major molecular targets of the mitochondrial respiratory chain unraveled by our study are complex I and cytochrome *c*. Both are modulated by concentrations of the flavonoids quercetin and kaempferol in the low micromolar range, i.e. by concentrations likely to be attained in the brain in studies showing strong neuroprotection by kaempferol against cerebral ischemia–reperfusion injury [85] and 3-nitropropionic acid-induced neurodegeneration in the brain striatum [18,86].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2011.09.022.

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