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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.