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(+)-Catechin inhibits heart mitochondrial complex I and nitric oxide synthase: functional consequences on membrane potential and hydrogen peroxide production

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In order to study the *in vitro* effect of flavan-3-ol (+)-catechin on the enzymatic activities of mitochondrial complex I and nitric oxide synthase (mtNOS), as well as the consequences on the membrane potential and H_2O_2 production rate, isolated mitochondria from rat heart were exposed to 3 nM to 100 μM (+)-catechin. NADH–Q_1 reductase (complex I) and mtNOS activities were inhibited 25% and 50%, respectively, by the addition of 10 nM (+)-catechin to the reaction medium. Moreover, in the nM range, (+)-catechin decreased state 4 mitochondrial membrane potential by about 10 mV, but failed to change the membrane potential measured in the presence of ADP. (+)-Catechin (10 nM) inhibited not only complex I activity, but also the H_2O_2 production rate (35%) sustained by malate–glutamate, in accordance with the decrease observed in mitochondrial membrane potential. Considering (+)-catechin concentrations lower than 10 nM, linear and positive correlations were obtained between mitochondrial complex I activity and either NO (r^2 = 0.973) or H_2O_2 production rates (r^2 = 0.958), suggesting a functional association among these parameters. Altogether, the results indicate that (+)-catechin, at nM concentrations, inhibits mitochondrial complex I activity, leading to membrane potential decline and consequently to reduction in H_2O_2 and NO production rates. The decrease in mtNOS activity could also be a consequence of the direct action of (+)-catechin on the NOS structure, this effect being in accordance with the functional interaction between complex I and mtNOS, as previously reported.

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Introduction

The heart is an organ dependent on energy to maintain normal contractile function, with mitochondria being the organelles that generate 95% of cellular ATP by oxidative phosphorylation. In this process, complex I (NADH-ubiquinone oxidoreductase) catalyzes the transfer of two electrons from NADH *via* flavin mononucleotide (FMN) and a series of iron–sulfur centers (Fe–S) to ubiquinone (UQ), in a reaction associated with proton translocation across the inner membrane, contributing to the proton-motive force.[1–3] Complex I is also a major source of reactive oxygen species, producing superoxide anion ($O_2^{\bullet-}$) through the autoxidation reaction of flavin-semiquinone ($FMNH^{\bullet}$)[4,5] or reduced FMN ($FMNH_2$),[6] as well as ubisemiquinone.[7] In the mitochondrial matrix, Mn-superoxide dismutase (Mn-SOD) dismutates $O_2^{\bullet-}$ to produce hydrogen peroxide (H_2O_2).[8–11] In addition, $O_2^{\bullet-}$ reacts with nitric oxide (NO), the latter species is produced by mitochondrial nitric oxide synthase (mtNOS), an isoenzyme of the NOS family located in the mitochondrial inner membrane.[12–14] The reaction between $O_2^{\bullet-}$ and NO yields the strong oxidant and nitrating compound, peroxynitrite ($ONOO^-$). When the steady-state concentration of $ONOO^-$ is increased, tyrosine nitration, protein oxidation and damage to Fe–S centers might take place,[15–17] leading to complex I inhibition. Dysfunction of complex I is found in clinical conditions such as Parkinson's disease,[18–21] ischemia–reperfusion,[22,23] endotoxic shock[24] and aging;[25–27] and it is usually accompanied by changes in mtNOS activity,[20,22–25] increases in H_2O_2 and $ONOO^-$ production rates and oxidative and/or nitrosative damage. This finding agrees with the reported functional interaction between complex I and mtNOS[28–31] and with the dependence of mitochondrial NO production on membrane potential.[30,32,33]

Many studies have shown that polyphenols attenuate the progression of diseases associated with oxidative stress and

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mitochondrial dysfunction.[34,35] It has been described that, within the cells, mitochondria can accumulate flavonoids.[36,37] Plant polyphenols – flavonoids and non-flavonoids – are reducing agents that function as antioxidants by virtue of the hydrogen-donating properties of their phenolic hydroxyl groups as well as by their transition metal-chelating abilities. The aromatic hydroxyl groups of flavonoids produce an antioxidant pharmacophore comparable to the chroman moiety of tocopherols. Indeed, a high reactivity of flavonoids with radicals such as O_2^-, hydroxyl radical ($HO^\bullet$) and NO has been reported.[38–40] However, the effectiveness of flavonoids as cellular antioxidants cannot be accounted for 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 stress.[41] Furthermore, the data from dietary bioavailability of flavonoids point out that the physiologic plasma concentration of total metabolites does not exceed 10 μM.[42] The bioavailability of flavonoids also depends on the dietary sources, and it has been shown that total flavonoid metabolites can reach plasma concentrations of up to 4.0 μM with an intake of 50 mg aglycone equivalents, except for isoflavones that can reach higher concentrations.[43] Thus, although many studies support the hypothesis that the protective effects of flavonoids are related to their intrinsic antioxidant properties, many other modes of action for catechins have been suggested.[44–46] Indeed, it has been previously reported that flavonoids can cause high inhibition of cellular reactive oxygen species production through inhibition of redox enzymes like NAD(P)H oxidases,[47,48] xanthine oxidase,[49] monooxygenases, cyclooxygenases and lipooxygenases,[50] as reviewed by Gutierrez-Merino *et al.*[51]

Considering that (+)-catechin, a flavan-3-ol present in green and black teas and many other plant foods, has shown beneficial effects on cardiovascular pathological processes, such as ischemia/reperfusion-induced tissue injury[52] and hypertension,[53] and that flavonoids can accumulate in mitochondria,[36,37] the aim of this work was to study the *in vitro* effect of (+)-catechin, in the nM to low μM concentration range, on heart mitochondrial complex I and mtNOS activities, as well as the consequences on the membrane potential and H_2O_2 production rate.

Materials and methods

Drugs and chemicals

The flavonoid (+)-catechin, recombinant nNOS (N3033, from rat brain) and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other reagents were of analytical grade.

Heart mitochondria isolation and mitochondrial membrane preparation

Sprague–Dawley female rats of 6–7 weeks of age (200–220 g) were housed in cages in an environmentally controlled facility at 25 °C. The animals were subjected to circadian light–dark cycles, fed standard rat chow, and provided water *ad libitum*. Animals were sacrificed under a CO_2 atmosphere; hearts were removed, washed and weighed. The procedures used in this study were approved by the Animal Care and Research Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires (CICUAL; 0027118/2017), and this investigation was in accordance with the International Guiding Principles for Biomedical Involving Animals (ICLAS).

The heart mitochondrial fraction was obtained from whole heart homogenates through differential centrifugation, using a Sorvall RC5C centrifuge (Sorvall-Instruments-Du Pont, Model RC5S). Hearts were homogenized in a glass-Teflon homogenizer in a medium consisting of 230 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.40 (MSTE) at a ratio of 1 g tissue per 9 ml of medium. The homogenate was centrifuged at 700*g* for 10 min to remove nuclei and cell debris, the sediment was discarded and the supernatant was centrifuged at 8000*g* for 10 min to precipitate mitochondria. The pellet was washed twice and resuspended in the same buffer.[54] This suspension consisted of mitochondria able to carry out oxidative phosphorylation. The whole procedure was carried out at 0–4 °C.

Mitochondrial membranes were obtained by three cycles of freezing and thawing of mitochondrial preparations and homogenized by passage through a 25-gauge hypodermic needle.[55]

Mitochondrial complex I activity

Complex I activity was studied following NADH–cytochrome c reductase (complex I–III) and NADH–Q_1 reductase (complex I) activities. Absorbance changes were monitored using a Beckman DU 7400 diode array spectrophotometer, at 30 °C.

In order to measure NADH–cytochrome c reductase activity, mitochondrial membranes suspended in 100 mM KH_2PO_4/K_2HPO_4, pH 7.40 (0.01–0.03 mg protein per ml) were pre-incubated for 2 min in the absence or in the presence of 1.0 to 50 nM of (+)-catechin. Then, 0.2 mM NADH, 25 μM cytochrome c^{3+}, and 0.5 mM KCN were added. Cytochrome c reduction was performed at 550–540 nm (ε = 19 mM^{-1} cm^{-1}) and the enzymatic activity was expressed as nmol cyt. c^{2+} per min per mg protein, taking into account the absorbance changes sensitive to rotenone (1 μM) addition.[56]

NADH–Q_1 reductase activity was measured in a medium consisting of 10 mM Tris-HCl, 1 mM EDTA, and 50 mM KCl, at pH 7.40. Mitochondrial membranes (0.01–0.03 mg protein per ml) were pre-incubated for 2 min in the absence or in the presence of 10 nM to 100 μM (+)-catechin. After that, 75 μM NADH, 50 μM 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (ubiquinone-1 or Q_1), 2 mM KCN, and 1 μM antimycin were added, and NADH consumption was carried out at 340–380 nm (ε = 5.5 mM^{-1} cm^{-1}). As the control, the effect of 1 μM rotenone was tested. Complex I activity was expressed as nmol NADH per min per mg protein.[57]

Nitric oxide synthase activity

Nitric oxide synthase activity was determined performing the oxidation of oxyhemoglobin (HbO_2) to methemoglobin at

577–591 nm (ε = 11.2 mM^{-1} cm^{-1}) using a Beckman DU 7400 diode array spectrophotometer, at 37 °C.[55,58]

The NO production rate by heart mitochondrial membranes, *i.e.* mtNOS activity, was measured in a reaction medium consisting of 100 mM KH_2PO_4/K_2HPO_4, pH 7.40, 1 mM $CaCl_2$, 100 μM NADPH, 10 μM dithiothreitol (DTT), 4 μM Cu, Zn-SOD, 0.1 μM catalase, 1 mM L-arginine, 20 μM HbO_2, and mitochondrial membranes (0.20–0.30 mg protein per ml) pre-incubated for 2 min in the absence or in the presence of 1.0 to 25 nM of (+)-catechin.

The NO production sustained by rat recombinant nNOS was measured in a reaction medium consisting of 50 mM HEPES, pH 7.40, 1 mM $CaCl_2$, 100 μM NADPH, 170 μM DTT, 10 μM tetrahydrobiopterin (BH_4), 1 μM calmodulin, 50 μM L-arginine, 5 μM HbO_2 and 0.05–0.18 units per ml nNOS. The effect of 3 and 10 nM of (+)-catechin was tested.[31]

In order to consider the NOS inhibitor-sensitive HbO_2 oxidation, control experiments adding 2 mM N^{ω}-monometil-L-arginine (L-NMMA) were performed. The absorbance changes that were inhibited by L-NMMA were expressed as nmol NO per min per mg protein or nmol NO per min per U.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was determined by measuring rhodamine 123 (Rh-123) fluorescence at 503–527 nm ($\lambda_{exc\text{-}em}$) with a Hitachi F-3010 spectrofluorometer, at 30 °C.[32,59,60] Rhodamine-123 was dissolved in ethanol and its concentration was assayed spectrophotometrically at 507 nm (ε = 101 mM^{-1} cm^{-1}). The ethanol concentration in the measuring medium was kept below 0.2% (v/v).

The fluorescence of the media containing 150 mM sucrose, 2 mM $MgCl_2$, 5 mM KH_2PO_4/K_2HPO_4, 30 mM KOH-HEPES, pH 7.40, and 0.1 μM Rh-123 was determined before addition of the mitochondrial suspension. This measurement was used as an indication of the total dye concentration ($[Rh\text{-}123]_{total}$, in nmol μl^{-1}). The heart mitochondrial suspension (0.10–0.20 mg protein per ml) was pre-incubated for 2 min in the absence or in the presence of 10 nM, 100 nM or 5.0 μM (+)-catechin and added to the reaction media. In order to establish resting respiration (state 4), 6 mM malate and 6 mM glutamate were added. State 3 respiration was achieved by the addition of 0.5 mM ADP. The hyperpolarization or depolarization of mitochondria was induced by 1 μM oligomycin or 1 μM m-CCCP addition, respectively. After an equilibration time, the fluorescence of the suspension was measured and the content of the cuvette was centrifuged at 15 000*g* to pellet mitochondria. The Rh-123 concentration remaining in the media ($[Rh\text{-}123]_{out}$, in nmol μl^{-1}) was calculated from the fluorescence values of the supernatant. The initial total amount of Rh-123 in the cuvette ($[Rh\text{-}123]_{total}$) and the amount remaining in the media ($[Rh\text{-}123]_{out}$) were used to calculate by subtraction the total amount of Rh-123 taken up by mitochondria ($[Rh\text{-}123]_{mit}$, in nmol per mg protein). The concentration of free Rh-123 in the matrix ($[Rh\text{-}123]_{in}$, in nmol μl^{-1}) was calculated using the following equation, and the binding partition coefficients at 30 °C are (K_i = 26 μl mg^{-1}, K_o = 120 μl mg^{-1}):[61]

$$[\text{Rh-123}]_{mit} = K_i\,[\text{Rh-123}]_{in} + K_o\,[\text{Rh-123}]_{out}$$

Mitochondrial membrane potentials (negative inside) were calculated by using the electrochemical Nernst–Guggenheim equation: $\Delta\Psi = 59 \log([Rh\text{-}123]_{in}/[Rh\text{-}123]_{out})$.

Mitochondrial hydrogen peroxide production

Mitochondrial H_2O_2 production was performed fluorometrically at 365–450 nm ($\lambda_{exc\text{-}em}$), at 30 °C, through the scopoletin-horseradish peroxidase (HRP) assay,[62] using a Hitachi F-3010 fluorescence spectrophotometer. The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, and 20 mM Tris-HCl, pH 7.40, added with 0.5 μM Cu, Zn-SOD, 1 μM HRP, 1 μM scopoletin and coupled mitochondrial suspensions (0.10–0.30 mg protein per ml) pre-incubated for 2 min in the absence or in the presence of 1–100 nM (+)-catechin. Mitochondrial H_2O_2 production rates were tested in state 4 using 6 mM malate and 6 mM glutamate as complex I substrates. A calibration curve was recorded using H_2O_2 (0.05–0.35 μM) as the standard, previously titrated spectrophotometrically at 240 nm (ε = 43.6 M^{-1} cm^{-1}) to express the fluorescence changes as nmol H_2O_2 per min per mg protein.

Protein concentration and statistics

Protein concentration was determined with the Folin reagent[63] using bovine serum albumin as the standard. Results included in tables and figures are expressed as means ± SEM of four or five independent experiments. Dunnet's t test was used to analyze the significance of differences between paired determinations. The 0.01 or 0.05 probability levels were used as a criterion for biological significance. Statistical analysis was performed using GraphPad Instat4 (GraphPad Software, La Jolla, CA, USA).

Results

Mitochondrial complex I activity

The effect of (+)-catechin on mitochondrial complex I was studied measuring NADH–Q_1 reductase (complex I) and NADH–cytochrome c reductase (complex I–III) activities (Fig. 1). NADH–Q_1 reductase activity was 25% inhibited by the addition of 10 nM (+)-catechin to the reaction medium. The maximal inhibitory effect of this polyphenol on NADH–Q_1 reductase activity was observed at 100 μM (+)-catechin; under this experimental condition, only 50% of complex I activity has been detected. As it was expected, the addition of 1 μM rotenone produced the almost complete (>95%) inhibition of complex I activity (*data not shown*). Taking into account that cytochrome c is a molecular target of flavonoids,[64] NADH–cytochrome c activity could not be an adequate technique to study the *in vitro* effect of (+)-catechin on complex I activity. Despite the fact that the inhibitory effect was not statistically significant and was less noticeable than when NADH–Q_1 reductase

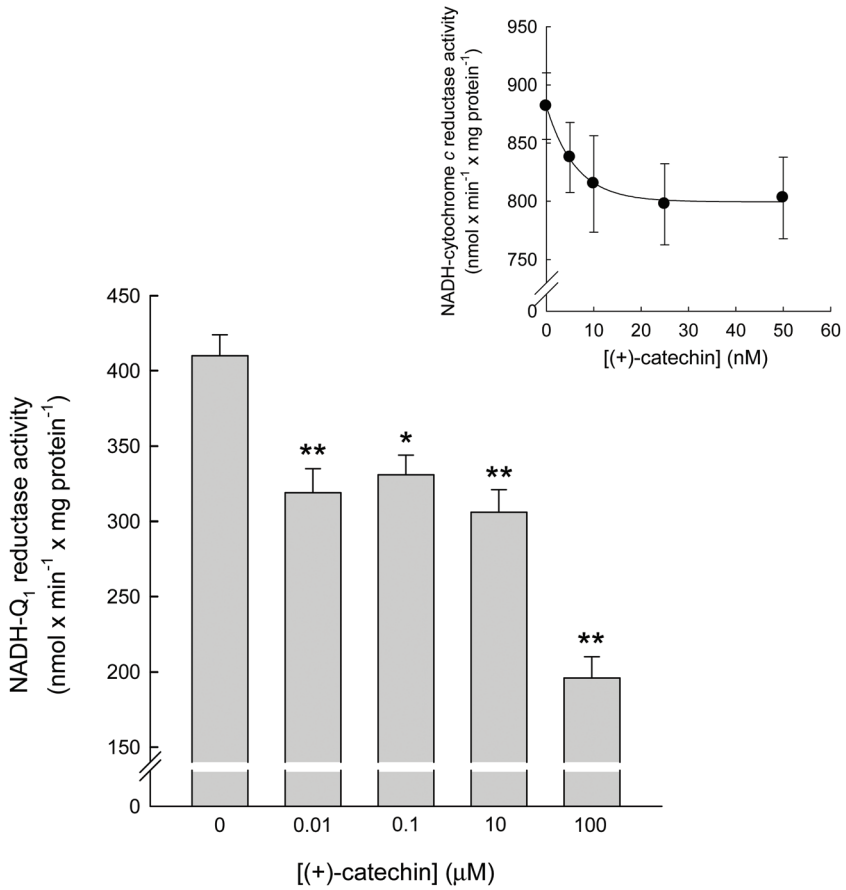

Fig. 1 Effect of (+)-catechin on mitochondrial complex I. NADH–Q_1 reductase (complex I) and NADH–cytochrome c reductase (complex I–III; inset) activities as a function of (+)-catechin concentration. Results are expressed as mean ± SEM of 5 independent experiments. *$p < 0.05$, **$p < 0.01$ in the presence *vs.* in the absence of (+)-catechin.

activity was measured, the influence of the addition of nM concentrations of (+)-catechin was also observed when complex I–III activity was measured (Fig. 1 inset): NADH–cytochrome c reductase activity was 10% reduced by 25 nM (+)-catechin.

Thus, in the nM range, (+)-catechin inhibited complex I measured not only by the NADH–Q_1 reductase assay but also by the NADH–cytochrome c reductase technique, suggesting an action of this flavan-3-ol on the complex I structure.

Mitochondrial membrane potential

In order to evaluate the effect of mitochondrial complex I inhibition by (+)-catechin on mitochondrial membrane potential ($\Delta\Psi$), the fluorescence changes associated with Rh-123 distribution were measured in the absence or in the presence of 10 nM to 5 μM (+)-catechin, using malate–glutamate as the substrate of complex I (Fig. 2). In the nM range, (+)-catechin decreased state 4 $\Delta\Psi$ (Fig. 2A) but failed to change the membrane potential measured in the presence of ADP (state 3; Fig. 2B). The membrane potential corresponding to state 4 respiration was reduced in 10 mV when mitochondria were exposed to 10 nM (+)-catechin. Although these changes were not statistically significant, the shift observed towards lower membrane potentials is highly relevant under physiological conditions. The physiological mitochondrial $\Delta\Psi$ is in a narrow range, between 150 (state 3) and 175 mV (state 4), where small changes in the potential are causative of depolarization. Thus, the difference in the membrane potential of mitochondria moving from a state 4 to a state 3 is not higher than 15–25 mV.[32] Membrane potential values detected during resting respiration, in the presence of 100 nM or 5 μM (+)-catechin, were similar to the one registered in the presence of 10 nM of this polyphenol, suggesting that this membrane potential-inhibition degree is the highest effect of this compound under the assayed experimental conditions. This observation is in agreement with the uncoupling effect exerted by classical uncoupling agents, such as FCCP or mCCCP.[5]

Hydrogen peroxide and nitric oxide production rates

Considering that complex I produces $O_2^{\cdot-}$, the stoichiometric precursor of H_2O_2, the effect of (+)-catechin on mitochondrial production of this latter species was measured using malate–glutamate as the complex I substrate. (+)-Catechin reduced state 4 mitochondrial H_2O_2 production (Fig. 3A) in a concentration dependent manner. At 10 nM (+)-catechin, the H_2O_2 production rate was reduced by 35%. The supplementation of

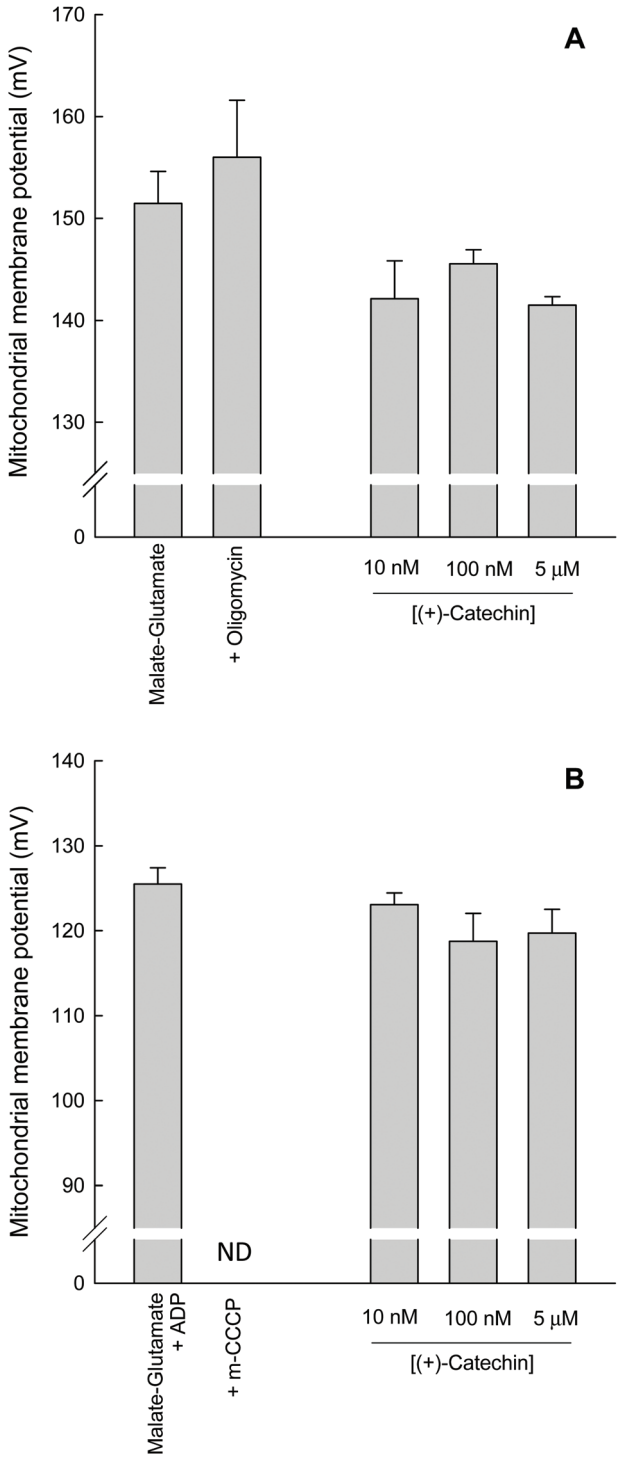

Fig. 2 Effect of (+)-catechin on mitochondrial membrane potential ($\Delta\Psi$) of mitochondria energized by malate–glutamate in the absence (A) and in the presence (B) of ADP. Hyperpolarization or depolarization of mitochondria was induced by 1 μM oligomycin or 1 μM m-CCCP addition, respectively, and was included as controls. Results are expressed as mean ± SEM of 5 independent experiments. ND: not detectable.

the reaction medium with 1 μM rotenone produced an enhancement in the H_2O_2 production rate of about 50% (0.72 ± 0.03 nmol per min per mg protein). When the mitochondrial suspension was pre-incubated in the presence of 5 μM (+)-catechin, H_2O_2 production was decreased by 40%, the same reduction degree that has been detected in the presence of 100 nM (+)-catechin, indicating that the inhibition observed at 100 nM was the maximal *in vitro* effect of (+)-catechin on H_2O_2 generation.

Taking into account the reported functional interaction between complex I and mtNOS activities,[28,31] and the regulation of mitochondrial NO production by membrane potential,[30,32,33] mtNOS activity was measured and the effect of 1.0–25 nM (+)-catechin was studied. This concentration range was selected according to the effects of that flavonoid on complex I activity and mitochondrial membrane potential. Fig. 3B shows that (+)-catechin inhibited NO production of heart mitochondrial membranes in a concentration dependent manner. Mitochondrial NOS activity showed a hyperbolic decay as a function of (+)-catechin concentration. At 10 nM (+)-catechin, a decline of about 50% in mitochondrial NO production was observed.

In addition, NO production by rat recombinant nNOS was assayed in an attempt to study the effect of (+)-catechin on the NOS structure. Table 1 shows that NO production was inhibited when nNOS activity was evaluated in the presence of 3 or 10 nM (+)-catechin, suggesting a direct action of this flavonoid on the enzyme structure. At 10 nM (+)-catechin, the recombinant nNOS activity was 45% decreased.

Moreover, considering the results obtained in the presence of (+)-catechin concentrations lower than 10 nM, linear and positive correlations were obtained (Fig. 3C) between mitochondrial NADH–Q_1 reductase activity and H_2O_2 production rate (r^2 = 0.958) and between NADH–Q_1 reductase and mtNOS activities (r^2 = 0.973), suggesting a functional association among these mitochondrial parameters.

Discussion

It has been reported that flavonoids can exert effects not only through *antioxidant* but also by *non-antioxidant* mechanisms, which can be combined to protect the cell against injury. Thus, flavonoids can act by virtue of their free radical scavenging properties, their transition metal-chelating abilities or by enzymatic activities modulation.[65,66] In the present work, the addition of nM concentrations of (+)-catechin to the reaction medium inhibited not only mitochondrial complex I activity (Fig. 1) but also the H_2O_2 production rate sustained by malate–glutamate (Fig. 3A), in accordance with the decrease observed in mitochondrial membrane potential (Fig. 2). These results agree with the finding of Lagoa *et al.*[64] who have shown that quercetin and kaempferol inhibit rotenone-sensitive NADH : Q_1 oxidoreductase activity in submitochondrial particles and the H_2O_2 production sustained by this complex, in rat brain mitochondria. These authors argue that the inhi-

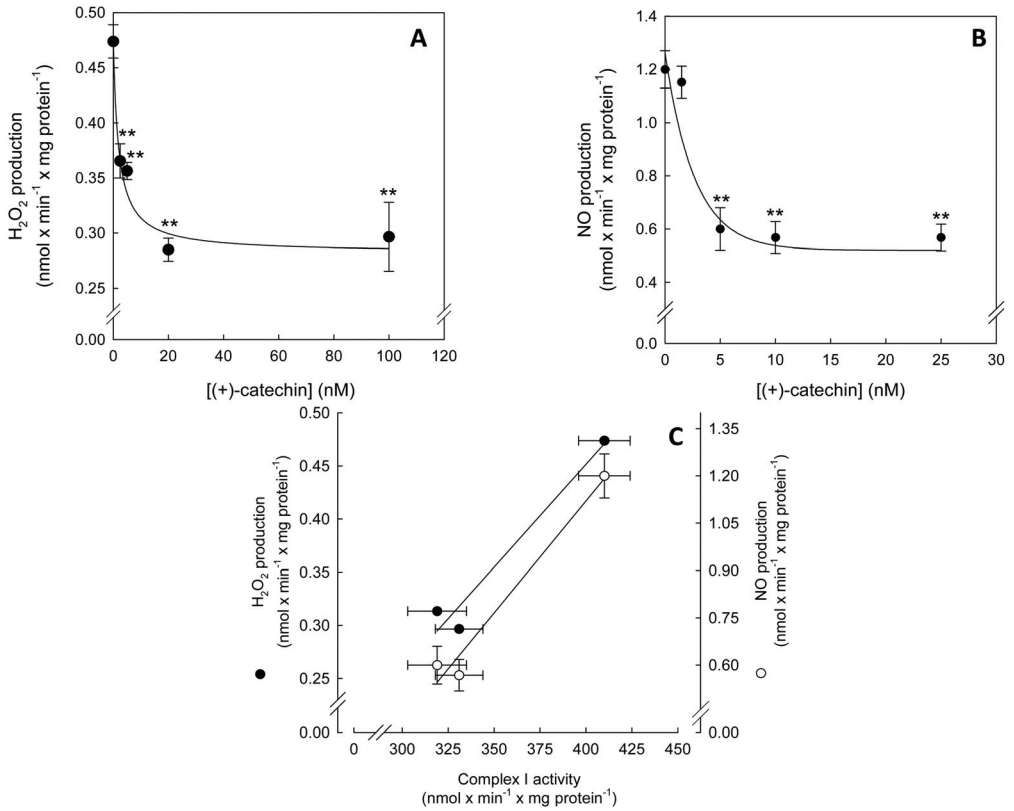

Fig. 3 Effect of (+)-catechin on mitochondrial H_2O_2 (A) and NO (B) production rates. Linear correlations between complex I and mtNOS activities (open circles), or complex I activity and H_2O_2 production rate (closed circles) (C) considering [(+)-catechin] concentration to be lower than 10 nM. Results are expressed as mean ± SEM of 5 independent experiments. $^{**}p < 0.01$ in the presence *vs.* in the absence of (+)-catechin.

Table 1 Effect of (+)-catechin on NO production sustained by recombinant nNOS activity

[(+)-Catechin] (nM)	nNOS activity (nmol NO min^{-1} U^{-1})
0.0	1.30 ± 0.10
3.0	0.67 ± 0.05**
10	0.71 ± 0.07**

Results are expressed as mean ± SEM of 4 independent experiments. $^{**}p < 0.01$ in the presence *vs.* in the absence of (+)-catechin.

bition of complex I activity depends on the concentration of ubiquinone added to the medium, suggesting that quercetin and kaempherol affect the binding of the coenzyme to the enzyme.[64] The inhibition of the electron transfer chain at complex I by kaempferol was also reported by Filomeni *et al.*,[67] who have shown that the pre-treatment of HeLa cells with 200 μM kaempferol inhibited malate–glutamate supported respiration, whereas no significant effect was observed in succinate supported respiration.

As a consequence of the inhibition of complex I activity exerted by (+)-catechin, the proton pumping by this complex could be affected, explaining the registered $\Delta\Psi$ reduction. It is important to point out that the state 4 $\Delta\Psi$ was 10 mV lower in the presence of the flavonoid than in its absence. According to Gerencser *et al.*,[68] this difference in the voltage value implies a 2-fold reduction in the maximal rate of mitochondrial ATP production in isolated mitochondria. In addition, it is known that the mitochondrial O_2^- production rate, *i.e.* of the stoichiometric precursor of mitochondrial H_2O_2, depends not only on the reduction degree of the components of the respiratory chain but also on the mitochondrial membrane potential.[8,10] It was recognized that ionophores, protophores and compounds that collapse $\Delta\Psi$ regulate the rates of H_2O_2 production.[8,10,54,69,70] Korshunov *et al.*[69] reported a hyperbolic relationship between membrane potential and H_2O_2 production in rat heart mitochondria with a threshold value that exceeds the state 3 $\Delta\Psi$ level, above which a very strong increase in H_2O_2 production takes place. Hansford *et al.*[71] showed that active H_2O_2 production by heart mitochondria requires both a high fractional reduction of complex I and a high membrane potential. According to Skulachev,[72] a slight uncoupling of mitochondrial respiration leads to a moderate decrease in membrane potential, preventing O_2^- and consequently H_2O_2 generation. Thus, nM concentrations of (+)-catechin could lead to a mild uncoupling of mitochondrial respiration sustained by substrates of complex I, the latter, the major entry point for electrons from NADH produced by the tricarboxylic acid cycle.

In addition, (+)-catechin inhibited NO production sustained by mtNOS. In the nM range, mtNOS activity showed a hyper-

bolic decay as a function of (+)-catechin concentration (Fig. 3B). Furthermore, when the effect of 10 nM (+)-catechin was tested on NO production catalyzed by recombinant nNOS, as a prototype of the NOS isoform located in mitochondria,[12] that flavonoid produced a decrease of about 45% in NO generation, suggesting that (+)-catechin could inhibit mtNOS activity through a direct action on the enzyme structure. Taking into account the quantity and the activity of the recombinant nNOS used (Table 1), it is possible to estimate the ratio between the concentration of the (+)-catechin added (3–10 nM) and the concentration of the pure enzyme. Accordingly, considering 200 U per mg protein from the data sheet of rat recombinant nNOS (N3033, Sigma Chemical Co.), and the maximal concentration of nNOS used under the assayed conditions, *i.e.* 0.18 U nNOS per ml, and 300 000 g mol^{-1} as MW of nNOS, the estimated concentration of nNOS in the reaction medium is ~3 nM. In this situation, the ratio (+)-catechin : recombinant nNOS is about 1 : 1. Therefore, the enzyme mtNOS is probably saturated when the effect of 1 to 25 nM (+)-catechin on NO production by heart mitochondria (nmol NO per min per mg total mitocondrial protein) is evaluated, because of that under this experimental condition the specific amount of mtNOS is expected to be lower than the quantity of recombinant NOS. This fact could explain why higher concentrations of (+)-catechin do not produce a greater inhibitory effect on mtNOS activity.

The combined decline in H_2O_2 and NO production rates is in agreement with the reported functional interaction between complex I and mtNOS proteins[22,23,29,31] and with the results obtained by Franco *et al.*[28] who have shown that complex I proteins from rat liver mitochondria co-immunoprecipitate with mtNOS. Moreover, data from our laboratory have shown that heart mitochondrial inside-out particles produce NO supported by succinate-dependent reversed electron flow in the respiratory chain, emphasizing the notion that mtNOS and complex I proteins are functionally associated[31] and contiguously located. The mtNOS and complex I association is compatible not only with the concept of respiratory chain supercomplex formation with strong protein–protein interactions,[73,74] but also with the dependence of mtNOS activity on the metabolic states and membrane potential. We have previously shown that mitochondrial NO production exponentially depends on mitochondrial membrane potential;[30,32,33] and this dependence is more pronounced in the physiological range of membrane potential (150–175 mV), where small changes in the $\Delta\Psi$ produce noticeable variations of mitochondrial NO release. Several studies have shown that abolishing $\Delta\Psi$ inhibits NO production by mtNOS activity, suggesting a tight regulatory interplay between mitochondrial membrane potential and NO production.[32,33,75] Therefore, in coupled mitochondria, the fall in $\Delta\Psi$ due to the addition of (+)-catechin could lead not only to the decrease in H_2O_2 production but also in NO generation by mtNOS.

Despite the emerging interest in the beneficial actions of polyphenols over mitochondria, there is little information regarding bioavailability and internalization of these bioactive compounds by the organelles. However, it has been shown that flavonoids can accumulate in mitochondria.[36,37,76] According to Stevens *et al.*[77] the physicochemical properties of polyphenols, such as lipophilicity and pK_a, actually favor their enrichment in mitochondria. Since (+)-catechin has favorable pK_{a1} (8.68 ± 0.23), close to the physiological pH of the cytosol and mitochondrial compartments, and distribution coefficient ($\log P$ = 0.61), it would able to reach the mitochondrial matrix and exert its biological effects within the organelle. According to our results, (+)-catechin, in the nM concentration range, inhibits mitochondrial complex I activity leading to a membrane potential decrease and consequently to H_2O_2 production reduction. The decrease in mitochondrial NO generation could be a consequence of the direct action of (+)-catechin on the NOS structure, and also correlates with the functional interaction between complex I and mtNOS and the dependence of mtNOS activity on $\Delta\Psi$ (Fig. 4).

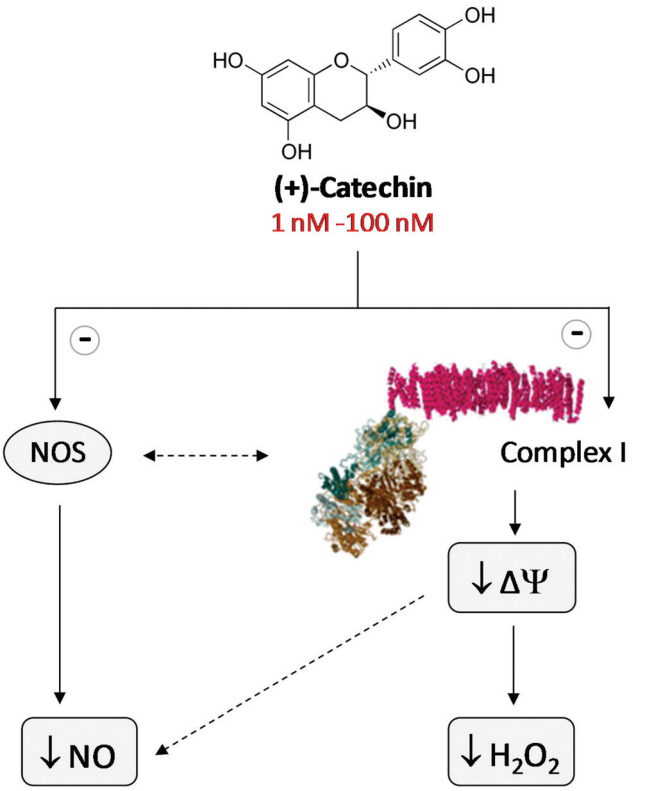

Fig. 4 Schematic representation showing the *in vitro* effects of nM to low μM concentrations of (+)-catechin on mitochondrial complex I activity, membrane potential ($\Delta\Psi$) and H_2O_2 and NO productions. Straight lines indicate pathways supported by the results included in this work and dotted lines suggest pathways reported by bibliography.

Conclusion

Physiologically relevant concentrations of (+)-catechin could modulate the mitochondrial redox state and the generation of signaling molecules, such as H_2O_2 and NO, through non-anti-

oxidant mechanisms. In this context, the mitochondrial enzymes complex I and mtNOS would be the direct targets of that flavonoid.

Abbreviations

H_2O_2	Hydrogen peroxide
mtNOS	Mitochondrial nitric oxide synthase
NO	Nitric oxide
$\Delta\Psi$	Mitochondrial membrane potential

Conflicts of interest

There are no conflicts to declare.

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