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Interaction of genistein with the mitochondrial electron transport chain results in opening of the membrane transition pore

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Abstract

Genistein, a natural isoflavone present in soybeans, is a potent agent in the prophylaxis and treatment of cancer. Addition of genistein to isolated rat liver mitochondria (RLM) induces swelling, loss of membrane potential and release of accumulated Ca^{2+} . These changes are Ca^{2+} -dependent and are prevented by cyclosporin A (CsA) and bongkreikic acid (BKA), two classical inhibitors of the mitochondrial permeability transition (MPT). Induction of the MPT by genistein is accompanied by oxidation of thiol groups and pyridine nucleotides. The reducing agent dithioerythritol and the alkylating agent *N*-ethylmaleimide (NEM) completely prevent the opening of the transition pore, thereby emphasizing that the effect of the isoflavone correlates with the mitochondrial redox state. Further analyses showed that genistein induces the MPT by the generation of reactive oxygen species (ROS) due to its interaction with the respiratory chain at the level of mitochondrial complex III.

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Keywords: Genistein; Mitochondria; Electron transport chain; Permeability transition

1. Introduction

Genistein, a natural isoflavone (Fig. 1), occurs in plants and, in particular, is a major component of soybeans. It has been shown to have anti-tumor, anti-oxidant and anti-inflammatory effects. Genistein is also able to modulate cell cycling and to prevent cancer in many different organ systems [1], most likely by attenuating cytokine-stimulated proliferation [2]. In addition, it has beneficial effects in treatment of chronic disorders such as osteoporosis and cardiovascular diseases and is also successfully used as an immunosuppressant [3,4]. Very recently, it has been reported that genistein is a potent inhibitor of α -glucosi-

dase [5]. This and other glucosidases are known to be involved in a variety of metabolic disorders and other diseases such as diabetes [6] and cancer [7] as well as in viral attachment [8]. It is believed that the consumption of large quantities of soy in Asian countries contributes to the low incidence of these chronic disorders and that it might play an important role in promotion of human health [4]. Many studies have identified numerous enzyme targets of its inhibitory action in living cells [3]. For example, genistein is a well-known inhibitor of tyrosine kinases [9], and many of its effects have been attributed to its alteration of tyrosine kinase-dependent signal transduction processes. Recently, it has been proposed that genistein induces apoptosis in RPE-J cells by provoking mitochondrial alterations characteristic of mitochondrial permeability transition (MPT) induction [10], a key phenomenon in cell death by apoptosis and necrosis [11,12]. This hypothesis contrasts with a study showing that several flavonoids are able to inhibit lipid peroxidation and consequently block the MPT according to their radical-scavenging action and/or antioxidant potential [13]. In reality, the behaviour of flavonoids is complex: they could act as either antioxidants or prooxidants, depending on their concentration,

Abbreviations: Ant. A, antimycin A; BKA, bongkreikic acid; CsA, cyclosporin A; DTT, dithioerythritol; MPT, mitochondrial permeability transition pore; NEM, *N*-ethylmaleimide; RLM, rat liver mitochondria; ROS, reactive oxygen species; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $\Delta\Psi$, membrane potential

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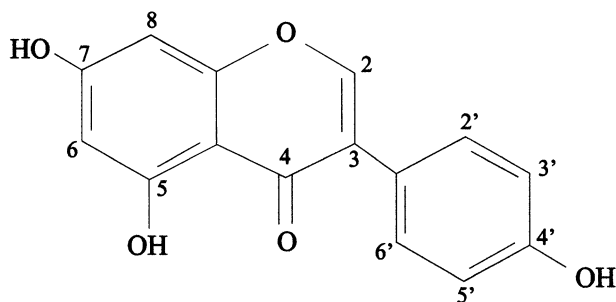


Fig. 1. The structure of genistein.

free radical source and presence of transition metals [14]. Indeed, some flavonoids can auto-oxidize in solution with the production of anion superoxide, hydrogen peroxide and hydroxyl radicals (ROS) [14–16].

The aim of this study was to elucidate the role of genistein as a possible inducer of the MPT and to gain information regarding its mechanism of action.

2. Materials and methods

2.1. Chemicals

Genistein was purchased from Calbiochem and dissolved in DMSO. All other reagents were of the highest purity commercially available.

2.2. Mitochondrial preparations

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM Hepes (pH 7.4) and 1 mM EDTA [17]; EDTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard [18].

2.3. Standard incubation procedures

Mitochondria (1 mg protein/ml) were incubated in a water-jacketed cell at 20 °C. The standard medium contained 200 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM succinate and 1.25 μ M rotenone. Variations and/or other additions are given with the individual experiments presented. The control assays contained the same volume of DMSO as those carried out with genistein; the final DMSO concentration was less than 0.2% (v/v) and did not affect the assayed activities.

2.4. Determination of mitochondrial functions

Membrane potential was calculated on the basis of the movements of the lipid-soluble cation tetraphenylphosphonium (TPP^+) through the inner membrane, measured using a TPP^+ -specific electrode prepared according to Kamo et al. [19]. Mitochondrial swelling was determined by the change in the absorbance of mitochondrial suspensions at 540 nm

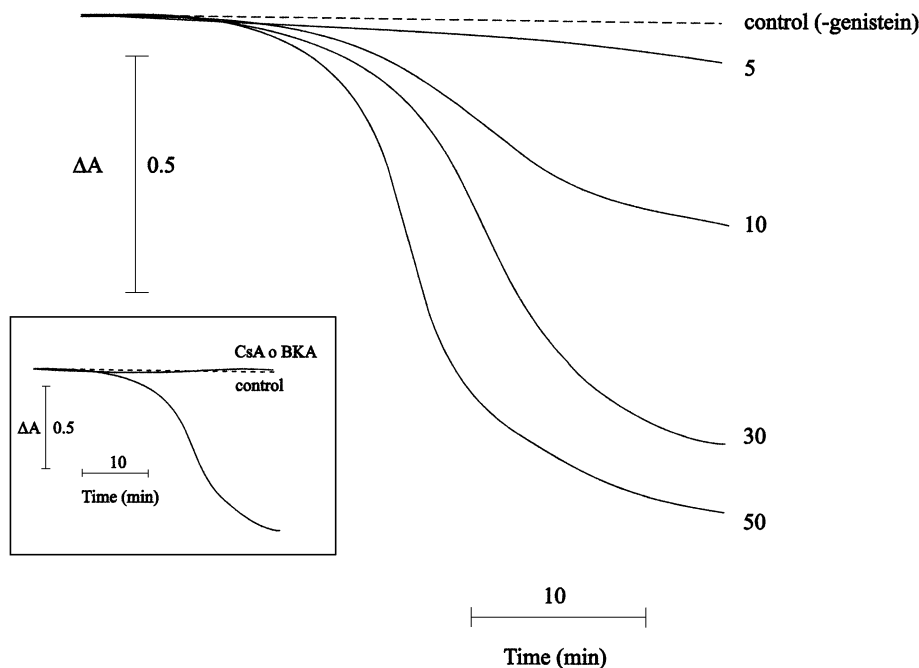


Fig. 2. Mitochondrial swelling induced by genistein in isolated RLM. RLM were incubated in standard medium supplemented with 30 μ M Ca^{2+} under the conditions indicated in Materials and methods. A downward deflection indicates mitochondrial swelling. Genistein was added at the concentrations (nmol/mg prot.) indicated to the right of the curves. The inset shows the effect of CsA (1 μ M) and BKA (5 μ M) on the swelling induced by 50 μ M genistein. The assays were performed seven times with comparable results.

using a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control. Ca^{2+} movements were followed using a Ca^{2+} -selective electrode (Radiometer F2112) and a calomel reference electrode (Radiometer K401). The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer with excitation at 354 nm and emission at 462 nm. In addition, for quantitative measurements and to exclude the possibility that the fluorescence

change could be influenced by the parallel decrease in light scattering, pyridine nucleotide oxidation was also determined by the methylethylketone method [20]. The protein sulfhydryl oxidation assay was performed as in Santos et al. [13].

The production of H_2O_2 in mitochondria was measured fluorometrically according to Matsumoto et al. [21]. In brief, mitochondria were incubated in the presence of horseradish peroxidase (9 U/ml) and 0.9 mM homovanillic

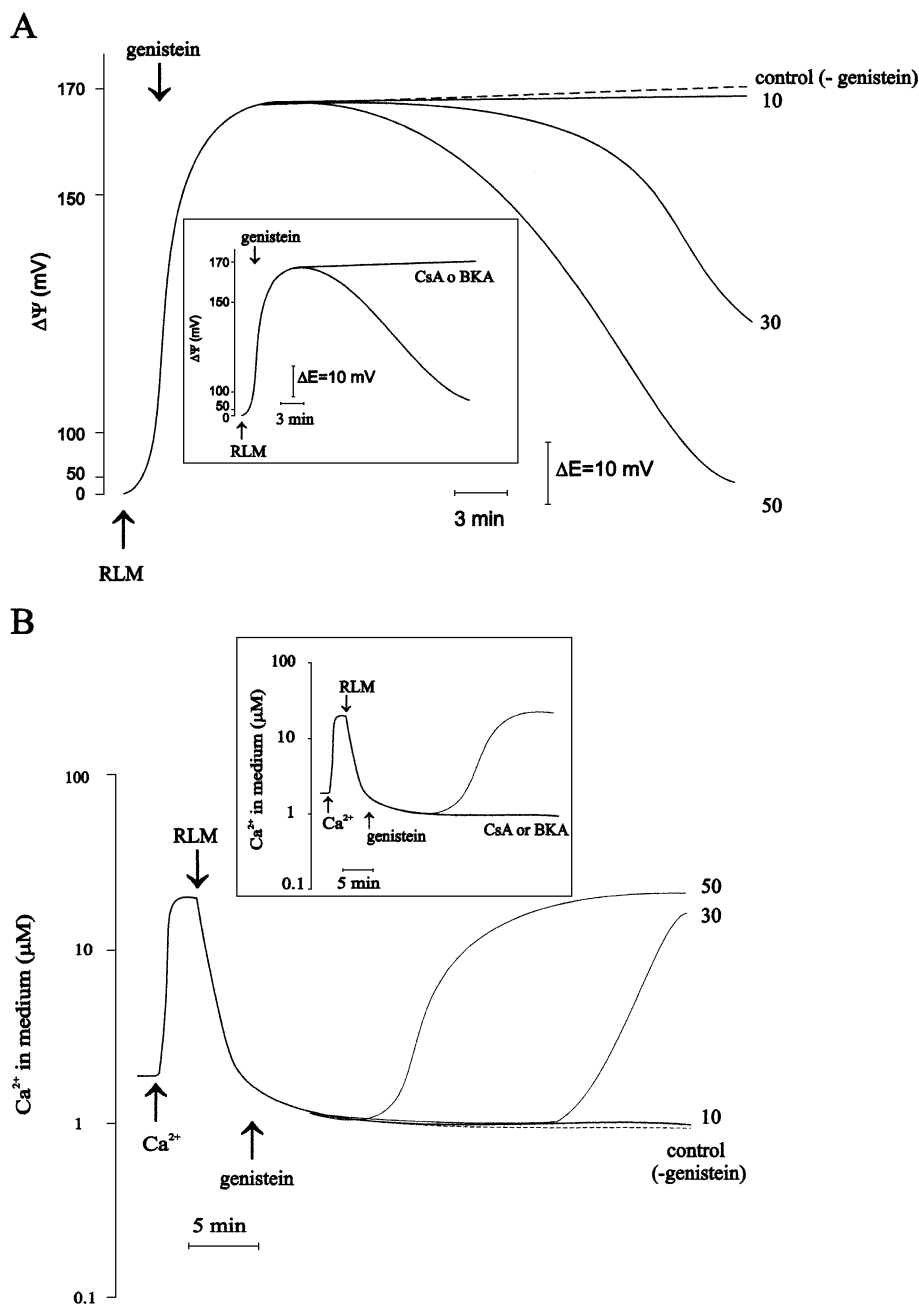


Fig. 3. Collapse of membrane potential (A) and Ca^{2+} efflux (B) induced by genistein. RLM were incubated as in Fig. 1 with genistein added at the indicated concentrations. The insets show the effect of 1 μM CsA and 5 μM BKA on $\Delta\Psi$ collapse (panel A) and Ca^{2+} efflux (panel B) induced by 50 μM genistein. ΔE =electrode potential. Five additional experiments exhibited the same trend.

acid. At intervals of time, aliquots of the incubation mixture were withdrawn and combined with 0.1 M NaOH. The fluorescence was then evaluated using an Aminco Bowman spectrofluorometer with excitation at 324 nm and emission at 426 nm. Fluorimetric calibration curves were prepared under the same experimental conditions using serial dilutions of H_2O_2 in the absence or presence of 1 mg/ml mitochondria.

3. Results

Addition of genistein to a mitochondrial suspension incubated in the presence of $30 \mu\text{M Ca}^{2+}$ provokes a dose-dependent change in the apparent absorbance that is indicative of large amplitude swelling (Fig. 2). The maximum rate and extent of swelling are obtained with a concentration of $50 \mu\text{M}$ genistein. No osmotic alteration is observable in the control curve obtained in the presence of $30 \mu\text{M Ca}^{2+}$ alone (dashed line). As observable in Fig. 3, mitochondrial swelling is accompanied by dose-dependent membrane depolarization (panel A) and release of accumulated Ca^{2+} (panel B). All these effects induced by $50 \mu\text{M}$ genistein are almost completely blocked by $1 \mu\text{M}$ cyclosporin A (CsA) and $5 \mu\text{M}$ bongkreikic acid (BKA), two typical inhibitors of the MPT (see insets in Figs. 2 and 3), suggesting that the mitochondrial changes are indicative of MPT induction.

The presence of EGTA, a calcium chelator, or ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, prevents the genistein-induced absorbance change (Fig. 4), suggesting that Ca^{2+} is necessary for induction of the MPT by genistein. In the absence of added Ca^{2+} , genistein induces swelling of low amplitude, which is completely prevented by EGTA or ruthenium red (Fig. 4). This observation suggests that genistein can induce the MPT, although to a

very reduced extent, by acting together with the contaminating Ca^{2+} in the medium ($2\text{--}3 \mu\text{M}$) and/or with the endogenous Ca^{2+} (7 nmol/mg prot.) which cycles across the inner membrane.

The redox state of mitochondrial thiols and pyridine nucleotides is a parameter strictly involved in MPT induction, either through an amplifying or triggering effect. The results reported in Fig. 5A show that the percentage of reduced mitochondrial thiols in the control condition (measured after 20 min of incubation), about 95% of the total, decreases to a value of 65% in the presence of $50 \mu\text{M}$ genistein, demonstrating a 30% increase in the oxidation of total thiols. Under the same conditions, pyridine nucleotides are also intensively oxidized (Fig. 5B). These oxidation phenomena are not observed in the presence of CsA or BKA, indicating that they are strictly correlated with induction of the MPT. However, as reported in Fig. 6, it must be emphasized that dithiothreitol (DTT) and *N*-ethylmaleimide (NEM) are also able to completely prevent mitochondrial swelling, thus confirming that a shift in the mitochondrial redox state towards a more oxidized level is implicated in the opening of the transition pore by genistein. These observations point out that genistein is able to directly or indirectly generate reactive oxygen species (ROS). In this regard, it is noteworthy that catalase exhibits only a very slight inhibitory effect, as shown in Fig. 6.

Due to their generation of incompletely reduced oxygen intermediates, e.g. superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH^\cdot) [6], mitochondria represent the main source of ROS production in the cell under physiological conditions. Therefore, we next investigated whether the interaction of genistein with the mitochondrial respiratory chain might result in increased ROS production. Fig. 7 shows that the addition of genistein to a mitochondrial suspension induces an increase in H_2O_2

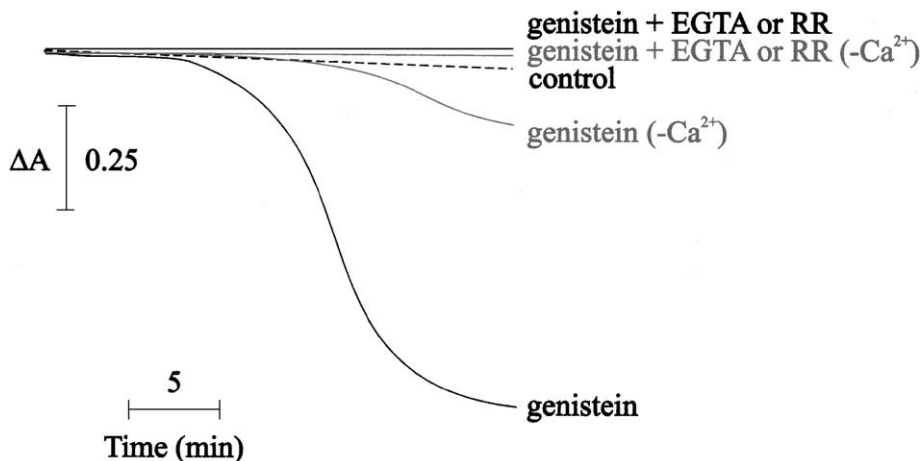


Fig. 4. Effect of EGTA and ruthenium red on swelling induced by genistein. Assays were carried out in standard medium in the absence ($-\text{Ca}^{2+}$) or presence of $30 \mu\text{M Ca}^{2+}$. Genistein ($50 \mu\text{M}$), EGTA (1 mM) and ruthenium red (RR, $0.5 \mu\text{M}$) were added as indicated. The assays were performed five times with comparable results.

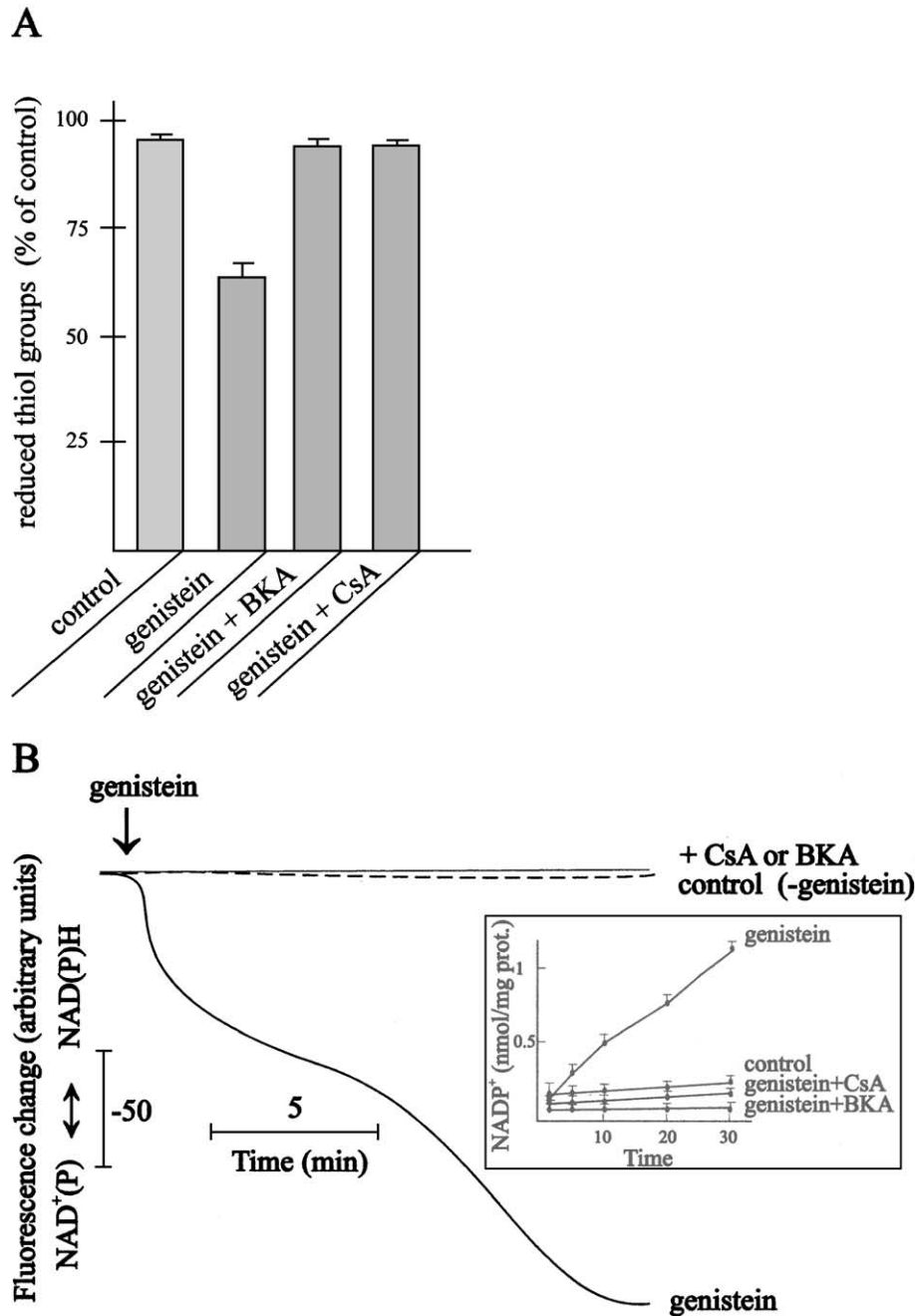


Fig. 5. Oxidation of mitochondrial thiols (A) and pyridine nucleotides (B) induced by genistein. RLM were incubated as in Fig. 1, with 50 μ M genistein, 1 mM EGTA, 1 μ M CsA or 5 μ M BKA added as indicated. Protein sulfhydryl oxidation was measured after 20 min of incubation. The mean values for thiol oxidation \pm S.D. from six experiments are reported (A). Six additional assays for pyridine nucleotide oxidation exhibited the same trend in fluorescence change (B). The inset reports a parallel quantitative determination of pyridine nucleotide oxidation performed by the methylethylketone method [20]. The mean values from three experiments \pm S.D. are reported.

formation up to 0.8 nmol/0.5 mg prot., thus supporting this hypothesis. In this regard, it must be pointed out that the addition of catalase has virtually no effect on the MPT (Fig. 6). These observations would indicate that H_2O_2 is not the ROS mainly involved in the MPT induced by genistein. Fig. 7 also shows that the increase in H_2O_2 formation by genistein is partially inhibited by CsA or BKA. This result

is explainable by taking into account the observation of Fig. 4 in which genistein is able to induce the MPT, albeit to a very low extent, by utilizing contaminating Ca^{2+} in the medium and/or the cycling of endogenous Ca^{2+} . The amount of H_2O_2 produced in the presence of CsA or BKA should be ascribable to the interaction of genistein with the respiratory chain. This amount would account for

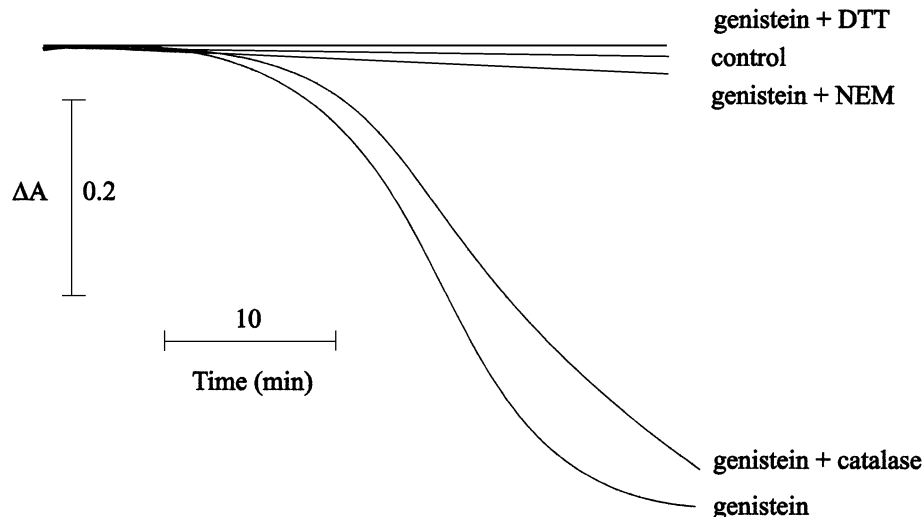


Fig. 6. Effect of DTT, NEM and catalase on mitochondrial swelling induced by genistein. RLM were incubated as in Fig. 1 with 50 μM genistein, 1 mM DTT, 10 μM NEM, 1000 U/mg prot catalase added as indicated. Shown is a typical experiment; five additional experiments yielded comparable results.

the low level of MPT induction. In this regard, it has been reported that the transition pore may have a number of intermediate conformations between fully closed and open [22] that are responsible for gradual changes in permeability of the inner membrane [23]. The production of H_2O_2 by genistein is not increased in the presence of 30 μM Ca^{2+} (results not reported), suggesting that the generation of other ROS is favoured due to the enhanced activity of the respiratory chain in this condition. This proposal is strongly supported by the negligible effect of catalase on the MPT observed in Fig. 6.

The results reported in Fig. 8 show the effects of different respiratory chain electron donors (3-hydroxybutyrate, succinate and ascorbate plus N,N,N',N' -tetramethyl-*p*-phenyldiamine (TMPD)) and inhibitors (rotenone and antimycin A) on the MPT induced by genistein. These

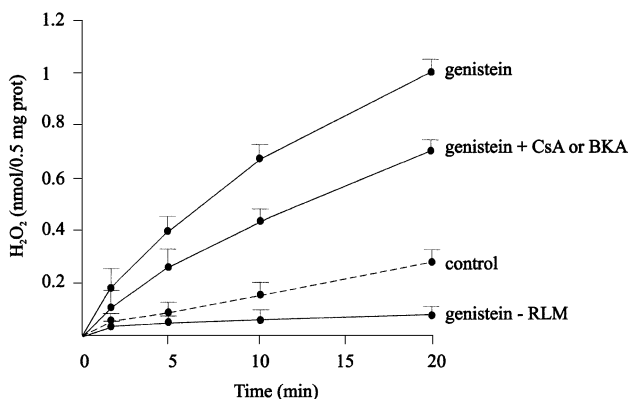


Fig. 7. Mitochondrial hydrogen peroxide production in the presence of genistein. RLM were incubated in standard medium in the presence of 0.9 mM homovanillic acid and 9 U/ml horseradish peroxidase with or without 50 μM genistein; 1 μM CsA or 5 μM BKA was added as indicated. The mean values from four experiments \pm S.D. are reported.

analyses permitted us to determine the effect of the respiratory chain components' redox state on the phenomenon and to identify the complex involved in the interaction with genistein.

We observed that genistein is able to induce the MPT in mitochondria energized by succinate plus rotenone in the presence of 3-hydroxybutyrate, which under these conditions strongly reduces the components of Complex I. The rate and extent of swelling (curve a, panel A) and the trend in $\Delta\Psi$ collapse (curve a, panel B) are identical to those observed in the absence of 3-hydroxybutyrate (curve b in panels A and B; also see Figs. 2, 4, and 6).

Addition of antimycin A to the 3-hydroxybutyrate-treated mitochondria blocks genistein-induced swelling (curve c, panel A). It is to be underlined that, by blocking electron flow through Complex III (cyt. bc_1 complex), antimycin A deenergizes mitochondria (curve c, panel B) and shifts the respiratory chain components lying upstream of its interaction site towards a completely reduced state.

Re-energization of these mitochondria by ascorbate plus TMPD (curve c', panel B) is ineffective in re-inducing the swelling phenomenon (curve c', panel A).

A different picture is observed when mitochondria are energized only by ascorbate plus TMPD instead of succinate plus rotenone. In this case, genistein is able to trigger the MPT both in the absence and presence of 3-hydroxybutyrate plus rotenone (see curves d and e, respectively, in both panels). The different time-course of MPT induction observed in these conditions compared with those of curves a and b is likely ascribable to differences in the Ca^{2+} threshold required to induce the phenomenon when mitochondria are energized with different respiratory substrates [24].

These results point out that MPT induction by genistein is completely prevented when Complex I and Complex III

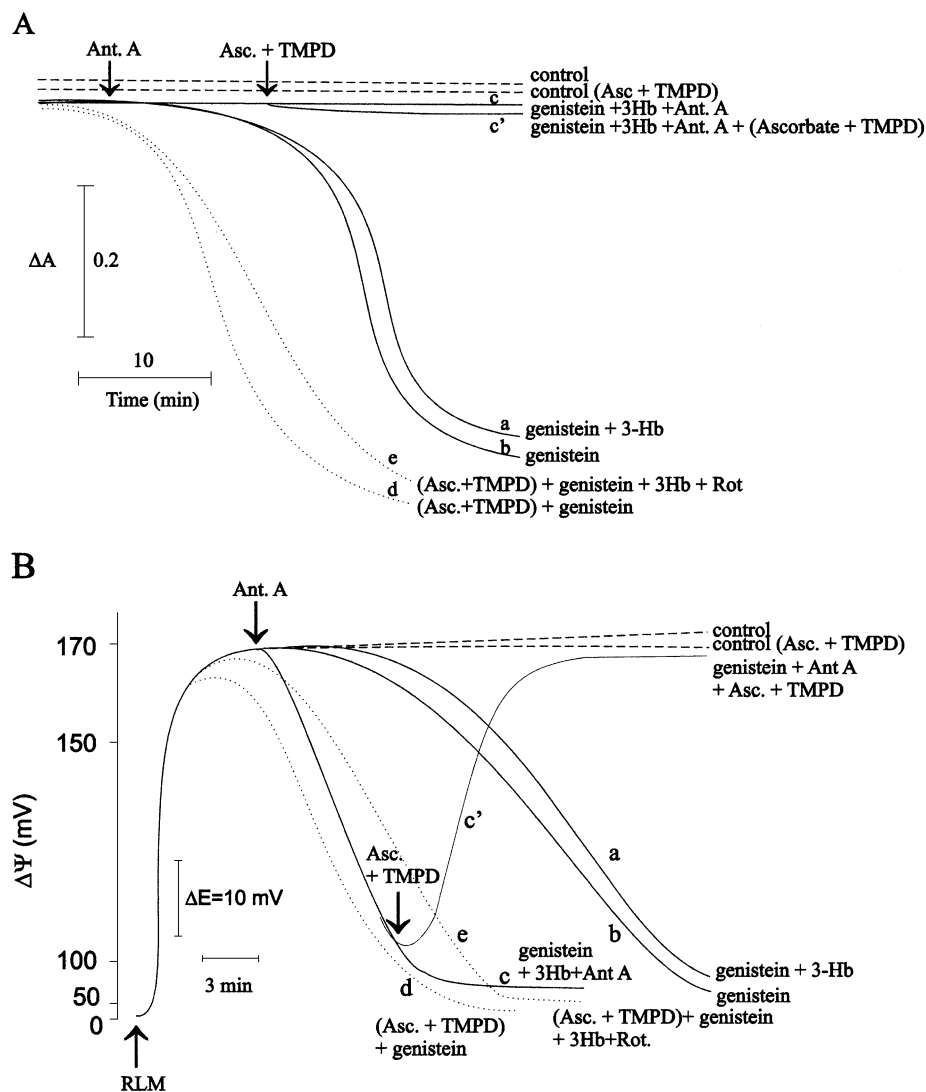


Fig. 8. Effects of different redox states of the respiratory chain complexes and of different membrane energizing substrates on mitochondrial swelling (A) and $\Delta\Psi$ (B) induced by genistein. Continuous lines refer to RLM incubated in standard medium in the presence of $30 \mu\text{M Ca}^{2+}$ (energization by succinate plus rotenone). Dashed lines refer to RLM incubated in standard medium deprived of succinate and rotenone and supplemented with ascorbate plus TMPD in the presence of $30 \mu\text{M Ca}^{2+}$ (energization by ascorbate plus TMPD). Fifty-micromolar genistein (gen.), 5 mM 3-hydroxybutyrate (3Hb), 10 mM ascorbate (Asc.) plus $100 \mu\text{M}$ TMPD, $1 \mu\text{M}$ antimycin A (Ant. A) and $1.25 \mu\text{M}$ rotenone (Rot.) were added as indicated. Controls refer to RLM energized with either succinate plus rotenone or ascorbate plus TMPD plus $30 \mu\text{M Ca}^{2+}$ and without any other additions. The assays were performed five times with comparable results.

(upstream of the inhibitory site of antimycin A) of the respiratory chain are in a reduced state.

4. Discussion

The results presented in this study show that treatment of RLM with a physiologically achievable concentration of genistein ($50 \mu\text{M}$, see Ref. [25]) results in dose-dependent matrix swelling, $\Delta\Psi$ collapse, release of pre-accumulated calcium and oxidation of thiol groups and pyridine nucleotides (see Figs. 2–6). All these events are inhibited by the endocapeptide immunosuppressant CsA, a ligand of mitochondrial cyclophilin [26], and by BKA, an inhibitor of the adenine nucleotide translocase [27] (Figs. 2 and 3); both

compounds are typical inhibitors of MPT induction [28]. Furthermore, EGTA and ruthenium red also exhibit a strong inhibitory effect on genistein-induced swelling, indicative of a strict Ca^{2+} dependence of the phenomenon (Fig. 4A). All the above-described experimental observations lead to the conclusion that genistein behaves as a typical MPT inducer.

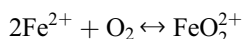
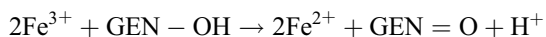
The oxidation of mitochondrial thiols and pyridine nucleotides reported in Fig. 5A,B is consistent with the involvement of oxidative stress in the phenomenon induced by genistein.

The proposed mechanism is that genistein acts as a prooxidant [14–16] producing ROS, most likely hydroxyl radicals, through its direct reaction with the transition metals of the respiratory chain and O_2 . In this regard, Cao et al. [14] have proposed a reaction sequence for ROS production

by flavonoids whose first step requires the oxidized state of the involved transition metal, which is reduced by reacting with the flavonoid in reduced form.

Two complexes of the respiratory chain were shown to be responsible for much of the ROS generated by mitochondria in physiological conditions [29–31] or in the presence of drugs [32–35]: Complex I, the NADH ubiquinone oxidoreductase, and Complex III, the ubiquinol-cytochrome *c* oxidoreductase.

The generation of hydrogen peroxide can be accounted for by the following reaction sequences [14]:



where GEN-OH is the reduced form of genistein, GEN=O is the oxidized form and $\text{Fe}^{3+/2+}$ is an Fe–S belonging to the involved respiratory complex. It is possible that genistein, after its oxidation by the respiratory chain, is reduced by diaphorase, a phenomenon previously demonstrated for menadione [36].

The results reported in Fig. 8 show that genistein is able to induce the MPT when mitochondria are energized with either succinate plus rotenone or with ascorbate plus TMPD.

In both these conditions, the presence of 3-hydroxybutyrate (plus rotenone when RLM are energized with ascorbate plus TMPD), which strongly reduces the components of Complex I, does not alter the effects of genistein. By taking into account the above-mentioned proposal that the first step of the genistein prooxidant mechanism involves the transition metal in the oxidized state [14], these results indicate that the Fe–Ss of Complex I are not involved in the interaction. Fig. 8 also shows that the addition of antimycin A to 3-hydroxybutyrate-treated, succinate-plus-rotenone energized RLM completely prevents MPT induction by genistein.

This observation is explained by taking into account the effect of the inhibitor on the respiratory chain. Antimycin A blocks electron flow between the b_{H} heme and, alternatively, the ubiquinone or ubisemiquinone anion at the Q_{n} (also called Q_{i}) site (see the Q cycle [37]), resulting in deenergization (Fig. 8B) and prevention of the MPT. It must be kept in mind that both the b_{L} and b_{H} hemes are reduced under these conditions. While subsequent addition of ascorbate plus TMPD to this assay reenergizes the RLM (Fig. 8B), the MPT does not occur. This result demonstrates that the components belonging to the bc_1 complex downstream of the site of antimycin A inhibition, i.e., Rieske protein and cyt c_1 , which have their transition metals in an oxidized state, are not involved in the phenomenon. Therefore, the target site must lie upstream of the site of antimycin A

inhibition in the segment comprising the b_{L} and b_{H} hemes. This proposal is further confirmed by the results obtained with RLM energized only by ascorbate plus TMPD. Under this conditions, the presence of 3-hydroxybutyrate plus rotenone, which reduces the four iron–sulfur clusters of Complex I but leaves the cyt bc_1 complex oxidized, does not affect the ability of genistein to promote the MPT.

All these observations demonstrate that genistein acts either on the Fe^{3+} of b_{L} or on the b_{H} heme of the cyt bc_1 complex. Given the standard redox potentials of b_{L} and b_{H} (–100 and +50 mV, respectively), and considering that the redox potential of flavonoids is in the range of –30 to +60 mV [16], we can hypothesize that the target of genistein is the Fe^{3+} of the b_{H} heme.

Previous papers reported that flavonoids can exhibit either antioxidant or prooxidant effects, depending on the presence of a source of ROS together with a transition metal. In this regard, genistein would appear to be peculiar in exhibiting only the prooxidant effect, as the swelling of RLM incubated in the presence of Ca^{2+} plus *tert*-butylhydroperoxide remains unaffected in the presence of genistein (results not reported).

The hydroxyl group of genistein involved in the ROS-producing reaction is most likely that in the 4' position (see Fig. 1), as the 5 and 7 hydroxyls lie in reciprocal unreacting *meta* positions.

The increase in H_2O_2 production in the presence of genistein reported in Fig. 7, while modest, is also compatible with the low or high amplitude swelling observed in the absence or presence of added 30 μM Ca^{2+} , respectively (see Fig. 4). In this regard, other observations have demonstrated that similar small amounts of H_2O_2 are able to induce the MPT [38,39]. The scarce protective effect exhibited by catalase on MPT induction (Fig. 6) raises some doubt about likelihood for direct intervention of H_2O_2 . One possible explanation is that H_2O_2 , which is generated in the proximity of the Q_{n} center near the internal side of the membrane, does not rapidly diffuse towards the outer site and cannot interact with catalase. Another possibility is that genistein favours the rapid formation of the highly reactive hydroxyl radical as also previously reported [14] and that this ROS is the main molecule responsible for the observed effects. The involvement of this radical has also been proposed for other inducers [40]. Although oxidative stress has long been known to be involved in MPT induction and/or modulation, its precise mechanism is not yet clear. Previous results suggested that the MPT occurs due to the direct oxidation of critical membrane thiol groups induced by respiratory chain-generated ROS [41–43] in the presence of Ca^{2+} . It has been proposed that two sites are the targets of oxidants and reductants [44]. The first site, named the “S” site, is a dithiol, while the second site, named the “P” site, is chemically undefined. The oxidation of these sites by small amounts of ROS produced by genistein would open the transition pore, while their reduction by DTE or NEM would favour its closure. The opening of the transition pore

results in substantial ROS production with consequent oxidation of the pyridine nucleotide pool and mitochondrial thiols [45]. This sequence of events explains the protective effects of DTE and NEM (Fig. 6).

Alternatively, ROS produced by genistein could induce an acceleration of Ca^{2+} cycling followed by the oxidation of pyridine nucleotides [46]. In this regard, it has been demonstrated that oxidized pyridine nucleotides are hydrolyzed by an NAD-hydrolase and the reaction product, ADP-ribose, opens a new specific pathway for Ca^{2+} efflux which enhances Ca^{2+} cycling. This phenomenon would further increase mitochondrial ROS formation [45] with catastrophic effects on the insulating properties of the inner membrane that would affect its bioenergetics capacity [45].

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