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Biochimica et Biophysica Acta 1658 (2004) 195–201

BIOCHIMICA ET BIOPHYSICA ACTA
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On the mechanism of mitochondrial permeability transition induction by glycyrrhetinic acid

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Received 23 March 2004; accepted 28 May 2004
Available online 21 June 2004

Abstract

Glycyrrhetinic acid (GE), the aglycone of glycyrrhizic acid, a triterpene glycoside which represents one of the main constituents of licorice root, induces an oxidative stress in liver mitochondria responsible for the induction of membrane permeability transition. In fact, GE, by interacting with the mitochondrial respiratory chain, generates hydrogen peroxide which in turn oxidizes critical thiol groups and endogenous pyridine nucleotides leading to the opening of the transition pore. Most likely the reactive group of GE is the carbonyl oxygen in C-11 which, by interacting mainly with a Fe/S centre of mitochondrial complex I, generates an oxygen-centered radical responsible for the pro-oxidant action.

Keywords: Mitochondria; Glycyrrhetinic acid; Electron transport chain; Reactive oxygen species; Permeability transition

1. Introduction

Licorice has long been known as a traditional herbal remedy and is used throughout the world [1].

One of the main components of licorice root is its principal water-soluble constituent glycyrrhizin, a pentacyclic triterpene derivative of the β-amirine type (oleanane). After oral administration or intravenous injection in humans and in experimental animals, glycyrrhizin is hydrolysed by glucuronidase of intestinal bacteria to its active principle aglycone, 18β-glycyrrhetinic acid (GE) (see Fig. 1), which is then absorbed into the blood [2]. Although much of the pharmacology focuses on glycyrrhizic acid and GE, it is important to recognise that licorice has many other components, such as flavonoids, that may have significant pharmacological effects [3].

Licorice extracts were primarily used as demulcents, expectorants, and mild laxatives. Recent research has shown that its constituents exhibit many pharmacological properties, including anti-inflammatory (cortisol-like), anti-hepatotoxic, anti-bacterial, anti-viral and anti-cancer effects [1,4].

Licorice also possesses many endocrine effects; in fact, it behaves as a mineralocorticoid, gluco- and antiglucocorticoid and exhibits estrogenic and anti-androgen effects [1].

In vivo and in vitro studies demonstrate that GE plays an important role in glucocorticoid metabolism inhibiting the enzyme 11β-hydroxysteroid dehydrogenase (11HSD; EC. 1.1.1.146). Two isoforms of the enzyme have been isolated, which are tissue-specific in humans. 11HSD type 1 (11HSD1) is predominantly a reductase, reactivating glucocorticoids from inert metabolites, while 11HSD type 2 (11HSD2) is predominantly a dehydrogenase and is located in the kidney and in other classical target tissues for aldosterone, where it inactivates cortisol to cortisone [5]. The subcellular distribution of 11HSD is greatest in mitochondria [6].

Recently, Armanini et al. [7] demonstrated that an extract of the root can decrease body fat mass, and the possible

Abbreviations: GE, glycyrrhetinic acid; MPT, mitochondrial permeability transition pore; RLM, rat liver mitochondria; ROS, reactive oxygen species; $\Delta\Psi$, membrane potential

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doi:10.1016/j.bbabio.2004.05.012

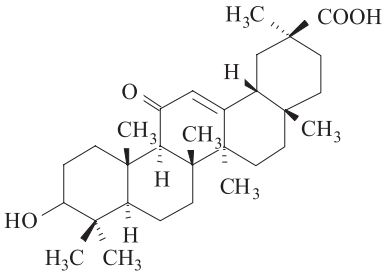


Fig. 1. Molecular structure of GE.

explanation of these findings is that licorice inhibits 11HSD1 at the level of fat cells. A block of this enzyme at the fat level reduces cellular cortisol and the disposition of fat [7].

The most common side effect of prolonged consumption of high amounts of these preparations is the hypokalemic hypertension. This side effect is due to an inhibition of 11HSD2, which inactivates cortisol to cortisone, thereby minimizing the binding of cortisol to mineralocorticoid receptors [8].

In vivo studies demonstrated that in mice GE administration induces cell death in thymocytes and splenocytes [9].

A very recent paper has demonstrated that GE is able to induce the Ca^{2+}-dependent permeability transition in liver mitochondria with the release of cytochrome *c* and AIF, suggesting a role for this substance as a pro-apoptotic agent [10]. These effects of GE have been attributed to oxidative stress induced by the substance, even if this has not yet been confirmed. The aim of this study is to give a clear demonstration of the pro-oxidant effect of GE and to determine its site of action. Another objective is to clarify the mechanism by which the interaction of GE with the mitochondrial membrane can generate the reactive oxygen species (ROS) responsible for opening of the pore.

2. Materials and methods

2.1. Chemicals

GE was purchased from Sigma and dissolved in absolute ethanol. 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 EGTA [11]; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard [12].

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 μM rotenone. Variations and/or other additions are described with the individual experiments presented. The control assays contained the same volume of ethanol as those carried out with GE; the final ethanol concentration did not exceed 0.1% (v/v) and did not affect the assayed activities.

2.4. Determination of mitochondrial functions

Membrane potential ($\Delta\psi$) was calculated on the basis of movement of the lipid-soluble cation tetraphenylphosphonium (TPP^+) through the inner membrane, measured using a TPP^+-specific electrode [13].

Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at 540 nm using a Kontron Uvikon mod. 922 spectrophotometer equipped with thermostatic control.

The protein sulfhydryl oxidation assay was performed as in the work of Santos et al. [14].

The production of H_2O_2 in mitochondria was measured fluorometrically by the scopoletin method [15] in an Aminco-Bowman 4-8202 spectrofluorometer.

3. Results

The histograms reported in Fig. 2 summarize the main effects of GE in inducing the mitochondrial permeability transition (MPT) as previously reported by Salvi et al. [10] in which it is stated that in the presence of Ca^{2+}, GE, at 10 μM concentration, is able to provoke $\Delta\psi$ collapse (A), mitochondrial swelling (B), increase in oxygen uptake (C) and oxidation of pyridine nucleotides (D). In the above-mentioned paper [10] it was suggested that GE might behave as a pro-oxidant agent in inducing the MPT, however, complete experimental evidence is not provided. In fact, the increased oxygen uptake and oxidation of pyridine nucleotides in the presence of Ca^{2+} (Fig. 2C and D) could represent the effects of transition pore opening rather than the cause of MPT induction. On the contrary, the observation of increased oxygen uptake with GE in the absence of Ca^{2+}, while less pronounced (panel B in Fig. 1 of Ref. [10]), might be due to a possible oxidative stress. The results in Figs. 3 and 4 aim at resolving this uncertainty. The histogram in Fig. 3 shows that GE alone is able to induce an oxidation of about 32% of the total content of reduced thiols. A lesser amount, approximately 13% of the total, is oxidized in the presence of Ca^{2+} alone, while the presence of both GE and Ca^{2+} induces the oxidation of 37%. These effects are concomitant with hydrogen peroxide generation, which is about 1.8 and 1.3

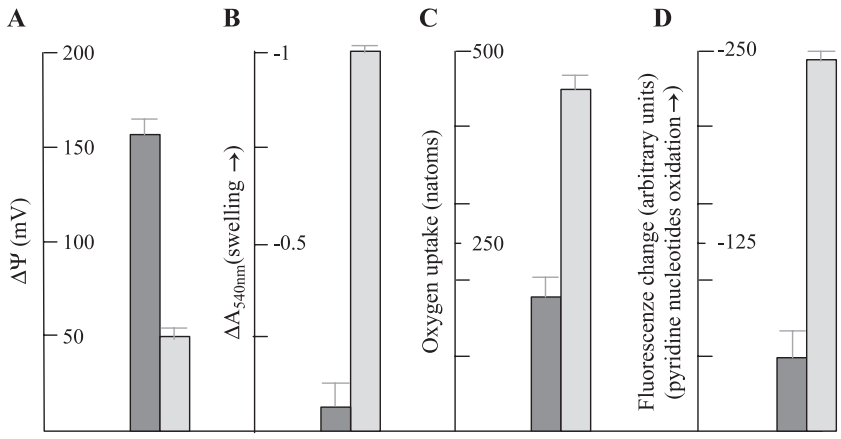


Fig. 2. Effect of GE on membrane potential (A); mitochondrial swelling (B); oxygen uptake (C); pyridine nucleotide redox state (D). Dark columns: controls; light columns: 10 μM GE plus 30 μM Ca^{2+}. RLM were incubated for 20 min, in standard medium as described in Materials and methods. Controls refer to experiments performed in the presence of either Ca^{2+} or GE alone. Mean values + standard deviation (S.D.) of four experiments are reported.

nmol/mg prot. in the presence or in the absence of Ca^{2+}, respectively (Fig. 4).

The observed generation of hydrogen peroxide and the oxidation of −SH groups induced by GE alone strongly suggest that the triterpenoid acts as a pro-oxidant. The results reported in Fig. 5 confirm this hypothesis. In fact, mitochondrial swelling induced by GE in the presence of Ca^{2+} is inhibited by ROS scavenger agents, both catalase and mannitol, and by the reducing agent dithioerythritol (DTE), although with different efficacy. It should be pointed out that these inhibitions are observed when the induction of the swelling has a rate comparable to or slower than that reported in the figure. A higher rate renders the effect of the inhibitors negligible. In order to determine the site(s) of action of GE, we took advantage of the fact that GE can interact with some transition metals of the respiratory chain in the oxidized state (see below) [16], and utilized a strategy previously applied in an investigation regarding the isoflavonoid genistein, which also induces an oxidative stress [17]. The aim of this strategy is to induce diverse redox levels on the respiratory complexes, so as to distinguish those involved in the interaction. The results reported in Fig. 6 show that RLM treated with GE in the presence of β-hydroxybutyrate, acting as an electron donor for complex I, exhibit large amplitude swelling (panel A, curve a) accompanied by a rapid $\Delta\psi$ collapse (panel B, curve a). If rotenone, which blocks β-hydroxybutyrate oxidation, and succinate, which reestablishes electron flux at the level of complex II and consequently complex III, are also present in the medium, mitochondrial swelling induced by GE is almost completely abolished (panel A, curve b) and $\Delta\psi$ remains almost unaffected (panel B, curve b). Curve c in Fig. 6A and B refers to the effects of GE in the presence of succinate plus

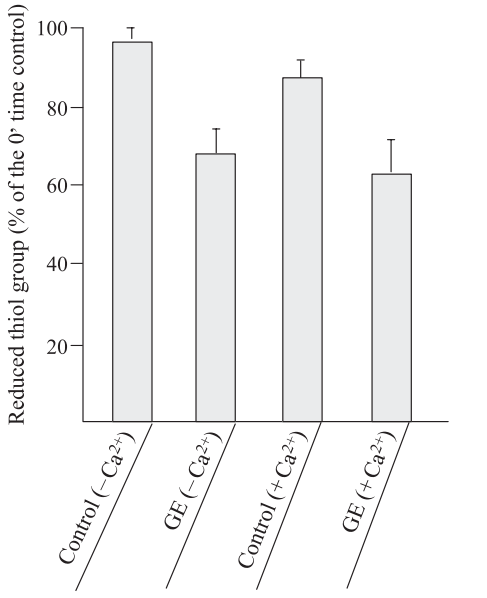


Fig. 3. Mitochondrial thiol oxidation by GE. RLM were incubated for 15 min in standard medium as indicated in Materials and methods. When present: 10 μM GE and 30 μM Ca^{2+}. Mean values + S.D. of five experiments are reported.

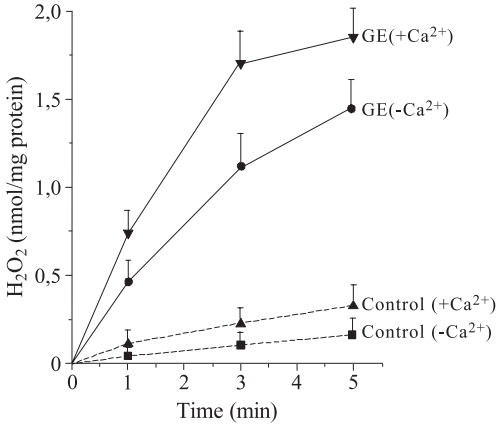


Fig. 4. Hydrogen peroxide generation by mitochondria incubated in the presence of GE. RLM were incubated as indicated in the legend to Fig. 3. Mean values + S.D. of five experiments are reported.

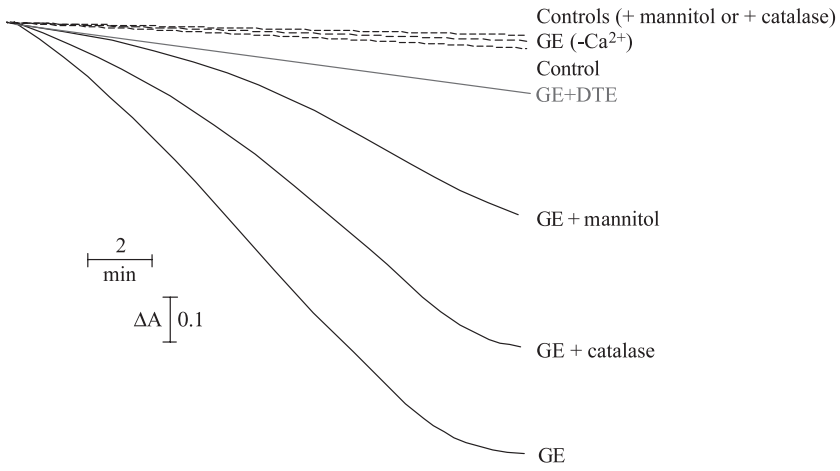


Fig. 5. Effect of ROS scavengers on mitochondrial swelling induced by GE. RLM were incubated in standard medium in the presence of 30 μM Ca^{2+}, except where indicated (− Ca^{2+}). When present: 10 μM GE, 100 μM mannitol, 500 U/mg prot. catalase, 2 mM DTE. The assays were performed four times with comparable results.

rotenone, a condition used in the preceding experiments (Figs. 2–5) and in a previous paper (see panels A and C in Fig. 1 of Ref. [10]), and has been presented here for a comparison with the other conditions. These results point out that MPT induction by GE is strongly prevented when complex I is in a completely reduced state.

The use of different energizing substrates and inhibitors, besides having to evaluate their effects on mitochondrial swelling and $\Delta\psi$ collapse induced by GE, also requires the measurement of parameters referring to the oxidative stress. The results reported in Fig. 7 show that β-hydroxybutyrate, in the absence of Ca^{2+}, induces the production of about 1.5 nmol/mg. prot. of hydrogen peroxide (panel A) and the oxidation of about 37% of the total content of thiol groups (panel B). If the substrate is succinate and incubation is performed also in the presence of β-hydroxybutyrate and rotenone, GE does not induce any significant changes on both H_2O_2 generation (A) and thiol oxidation (B). Glutathione oxidation by GE exhibits the same trend as thiol oxidation (results not reported).

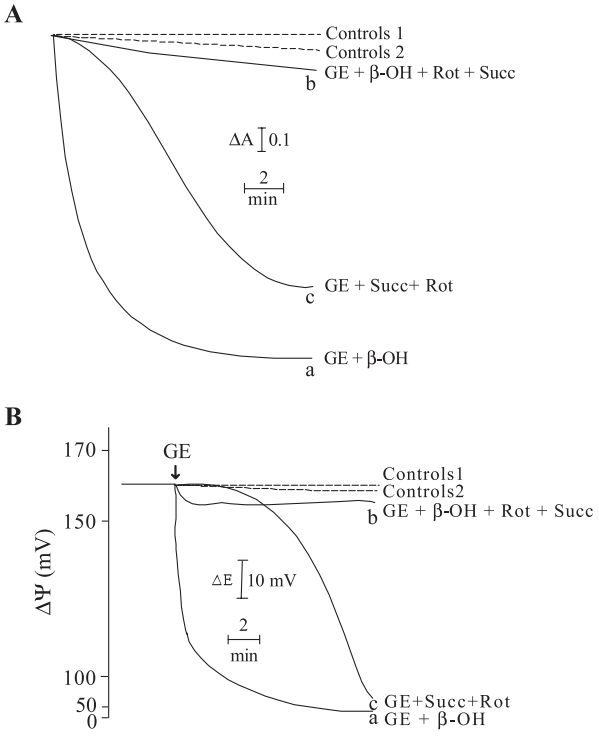


Fig. 6. Effect of different redox states of the respiratory chain complexes and of different membrane energizing substrates on mitochondrial swelling (A) and $\Delta\psi$ collapse (B) induced by GE. All the incubations were carried out as indicated in standard incubation procedures in the presence of 250 mM sucrose, 10 mM HEPES (pH 7.4), 30 μM Ca^{2+} (except for controls 2) and 10 μM GE (except for controls 1). Where indicated, 5 mM β-hydroxybutyrate (β-OH), 5 mM succinate (Succ), and 1.25 μM rotenone (Rot) were present. Controls 1 and 2 contain 5 mM β-OH or 5 mM Succ plus 1.25 μM Rot or 5 mM Succ plus 5 mM β-OH plus 1.25 μM Rot.

4. Discussion

The results presented in this investigation demonstrate that the MPT induced by GE in liver mitochondria, as previously reported [10] (see also Fig. 2), is due to an oxidative stress provoked by the interaction of the triterpenoid with the respiratory chain in the presence of Ca^{2+}. As shown in Fig. 4, GE alone produces hydrogen peroxide, which most likely is responsible for the oxidation of critical thiols (Fig. 3) leading to pore opening when Ca^{2+} is also present. It should also be pointed out that Ca^{2+} alone is able to produce H_2O_2, although to a much lesser extent than GE (Fig. 4), with a consequent oxidation of some thiol groups (Fig. 3). The presence of Ca^{2+} plus GE results in an oxidation of –SH groups that seems to sum the two effects, suggesting that GE and Ca^{2+} provoke the oxidation of different thiols. Since Ca^{2+} and GE alone are not able to induce the MPT, it seems clear that the phenomenon is triggered by the interaction of Ca^{2+} with adenine nucleotide

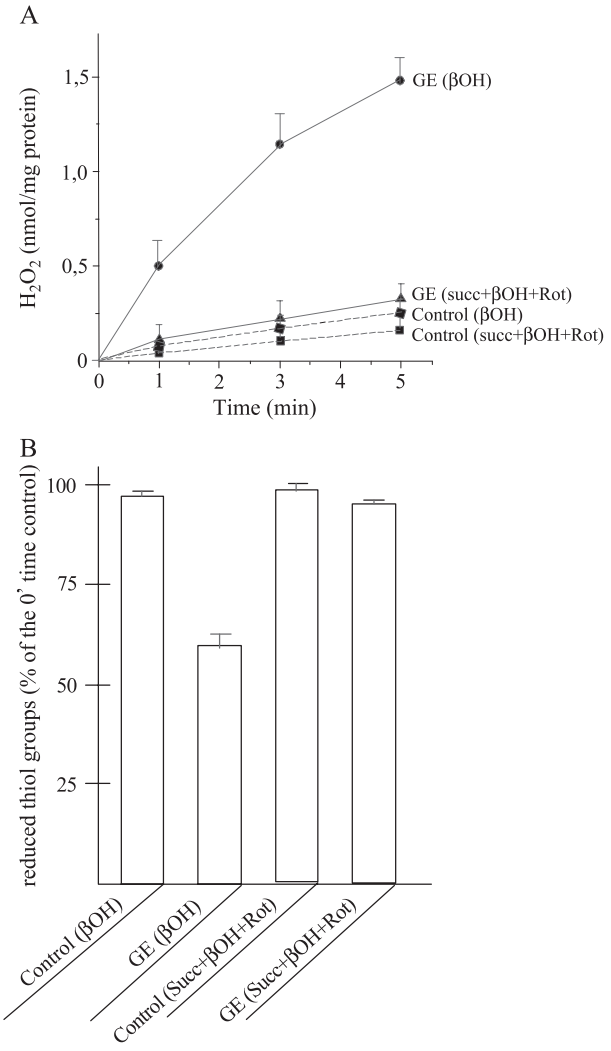
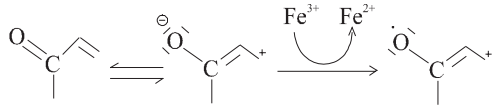


Fig. 7. Hydrogen peroxide generation (A) and thiol group oxidation (B) induced by GE in the presence of two different redox states of the respiratory chain complexes and of different membrane energizing substrates, and in the absence of Ca^{2+}. RLM were incubated as described in standard incubation procedures in the same medium described in the legend to Fig. 6, in the absence of Ca^{2+}. Where indicated, 10 μM GE, 5 mM β-hydroxybutyrate (β-OH), 5 mM succinate (succ), and 1.25 μM rotenone (Rot) were present. The controls refer to incubation in the absence of GE. Mean values + S.D. of four experiments are reported.

translocase, generally known as the main protein involved in the phenomenon, provided that some critical thiols of the transporter are oxidized [18]. The generation of hydrogen peroxide observed in the presence of Ca^{2+} alone is most likely attributable to its interaction with the anionic head of cardiolipin, which provokes some alterations in membrane organization. This may affect coenzyme Q mobility and favour ROS production [19]. It should also be pointed out that some mitochondrial preparations do not exhibit any effect with Ca^{2+} alone. If we evaluate the induction of MPT produced by the observed oxidative stress (see results in this paper and Ref. [16]), we note that the generation of hydrogen peroxide by GE seems to follow the same pathway previously observed with genistein [17]. However, it must be stressed that while the reactive group of genistein is the hydroxyl group in the 4′ position [17], the reactive site of GE, as also determined for some compounds structurally related to uric acid such as caffeine, theobromine and xanthine [20], is the conjugated carbonyl oxygen at C-11, which can be involved in keto-enol tautomerism.

This group would interact with a different mechanism than genistein with some transition metal of the respiratory chain in the oxidized form (most likely some Fe^{3+}) by producing a GE-derived oxygen centered radical by means of the following reaction also proposed by others [20]:



Then the centered oxygen radical would react with O_2 to form the superoxide anion $O_2^{\cdot -}$ which in turn generates hydrogen peroxide. Finally, H_2O_2, by interacting with Fe^{2+}, produces the hydroxyl radical, $OH^{\cdot}$, by means of the Fenton reaction (see Fig. 8). The possible involvement of conjugated carbonyl oxygen interacting with Fe^{3+} to produce ROS is also deducible in evaluating the MPT induced by the pigment curcumin [21]. The involvement of ROS in inducing the MPT is strongly supported by the results reported in Fig. 5, which shows that mannitol, a scavenger of hydroxyl radicals, is able to induce an inhibition of the phenomenon. This inhibition emphasizes that most of the MPT induction is due to the effects of $OH^{\cdot}$. Most likely the incomplete inhibition is due to the effect of H_2O_2 which can directly oxidize the critical thiol groups by the glutathione peroxidase/glutathione reductase system. The slight inhibitory effect observed with catalase is probably due to the location and orientation of the key site of ROS generation. If superoxide anion is produced in the bulk of the membrane, away from the outer surface and orientated towards the matrix, it is difficult for all the produced H_2O_2 to diffuse towards the external side to interact with catalase, hence the protective effect of the enzyme is relatively low. A strong inhibition, instead, is observable with DTE, which exhibits a broader antioxidant effect (Fig. 5). It should be pointed out that both mannitol and DTE, even though impermeable to the membrane, can penetrate the inner compartment and exhibit their action as soon the transition pore begins to open.

The results reported in Fig. 6 show that the maximum extent of MPT induction is obtained during the oxidation of β-hydroxybutyrate (curves a). This effect is almost completely abolished by the addition of rotenone and succinate demonstrating that the main target of GE is a Fe^{3+} of some Fe–S centre of complex I. Considering that the redox couple of GE is of the ubiquinone/semiquinone type, whose normal redox potential is about −160 mV [22], it is hypothesized that N-2 is the interacting centre. In fact, it

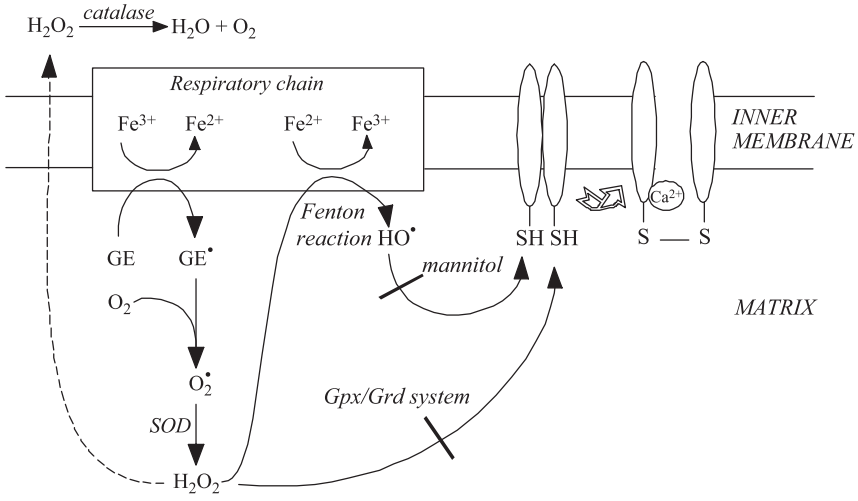


Fig. 8. Scheme summarizing the proposed molecular processes by which GE induces MPT pore opening. The centered oxygen radical GE· produced by the interaction of GE with complex I generates the superoxide radical $O_2^{\cdot -}$. $O_2^{\cdot -}$ is removed by superoxide dismutase (SOD), which promotes the generation of hydrogen peroxide (H_2O_2). In the presence of Fe^{2+}, H_2O_2 generates the highly reactive hydroxyl radical ($OH^{\cdot}$) that oxidizes −SH groups leading to pore opening in the presence of Ca^{2+}. Thiol oxidation and induction of pore opening can be provoked also by H_2O_2 via glutathione peroxidase/glutathione reductase system.

has a redox potential in the range of −20 to −160 mV [22].

The decreased amplitude of MPT observed with succinate plus rotenone (curves c) might be attributed to the interaction with N-2 whose iron is in a slightly reduced state due to the block of rotenone on the low electron flux induced by NAD-dependent endogenous substrates.

The results of Fig. 7, which show that GE, in the presence of β-hydroxybutyrate and absence of Ca^{2+}, induces H_2O_2 generation (A) and thiol oxidation (B), are indicative for the induction of an oxidative stress also in this condition. This effect is responsible for pore opening when Ca^{2+} is present (Fig. 6A and B, curves a). The amounts of these reaction products are higher than those observed with succinate plus rotenone (see Figs. 3 and 4) and account for the different extent of MPT induced in both these conditions (compare curves a and c in Fig. 6A and B). If mitochondria are energized by succinate in the presence of β-hydroxybutyrate plus rotenone, H_2O_2 generation and thiol oxidation by GE are negligible (Fig. 7A and B) and account for the almost complete prevention of MPT observed in this condition (Fig. 6A and B, curves c).

In conclusion, it should be emphasized that GE, depending on its targets, can behave as an anti- or pro-oxidant agent as observed with the isoflavonoid genistein [17], the alkaloid caffeine and its derivatives [20], and the natural yellow pigment curcumin [21]. The pro-oxidant behaviour is induced when the triterpenoid, similar to the cited substances, interacts with the transition metals, in particular the Fe^{3+} of the mitochondrial respiratory chain. This could represent a general mechanism induced by different reactive groups and different targets, with which several substances are able to exhibit both anti-oxidant and pro-oxidant properties and can collapse the bioenergetic capacity of mitochondria with the opening of the MPT pore. This may also be the initial step by which GE induces apoptosis of tumour cells [9,23].

Acknowledgements

The authors are grateful to Mr. Mario Mancon for his skilled technical assistance.

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