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Biochemical Pharmacology 66 (2003) 2375–2379
Short communication
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Glycyrrhetinic acid-induced permeability transition in rat liver mitochondria

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Received 24 June 2003; accepted 19 August 2003

Abstract

Glycyrrhetinic acid, a hydrolysis product of one of the main constituents of licorice, the triterpene glycoside of glycyrrhizic acid, when added to rat liver mitochondria at micromolar concentrations induces swelling, loss of membrane potential, pyridine nucleotide oxidation, and release of cytochrome *c* and apoptosis inducing factor. These changes are Ca^{2+} dependent and are prevented by cyclosporin A, bongkrekic acid, and *N*-ethylmaleimide. All these observations indicate that glycyrrhetinic acid is a potent inducer of mitochondrial permeability transition and can trigger the pro-apoptotic pathway.

Keywords: Glycyrrhetinic acid; Mitochondria; Permeability transition

1. Introduction

It is well established that licorice is still appreciated as a medicinal root and that the history of this plant as a pharmacological remedy dates back far into the past [1]. One of the main constituents of licorice root is glycyrrhizic acid (GI), a triterpene glycoside. In humans and in experimental animals, GI when administered orally, is hydrolyzed by intestinal bacteria to its aglycone 18β-glycyrrhetinic acid [2,3]. Other important constituents of the licorice root are flavonoids and isoflavonoids, that principally act as antioxidants [4]. Licorice extracts have many endocrine and biological properties [1], and recently it has been used in treatment of viral infections, like hepatitis, HIV, and chronic fatigue [5–8]. In addition, it has also some estrogen-like properties and reduces serum cholesterol [1]. Recently, Armanini *et al*. have demonstrated that an extract of the root can decrease serum testosterone [9] and body fat mass [10].

Glycyrrhetinic acid (GE) also blocks both isoforms (types I and II) of the enzyme 11β-hydroxysteroid dehydrogenase (11HSD; EC. 1.1.1.146) which is responsible for the interconversion of the hormonally active 11β-hydroxyglucocorticoids to their inactive 11-keto metabolites [11]. The subcellular distribution of 11HSD is greatest in mitochondria [12].

In vivo studies demonstrated that in mice GE administration induces cell death in thymocytes and splenocytes. These studies suggest that GE-induced apoptosis might be due to inhibition of liver glucocorticoid-metabolizing enzyme 11HSD type I [13,14]. The aim of this study was to evaluate the role of GE as a possible inducer of mitochondrial permeability transition (MPT), a key phenomenon in cell death and necrosis.

2. Materials and methods

2.1. Chemicals

18β-Glycyrrhetinic acid was purchased from Sigma and dissolved in absolute ethanol. Mouse monoclonal antibody

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Abbreviations: AIF, apoptosis inducing factor; BKA, bongkrekic acid; Cyt *c*, cytochrome *c*; GE, glycyrrhetinic acid; MPT, mitochondrial permeability transition; NEM, *N*-ethylmaleimide; RLM, rat liver mitochondria; TPP^+, tetraphenylphosphonium; $\Delta\psi$, membrane potential.

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doi:10.1016/j.bcp.2003.08.023

anti-cytochrome *c* (Cyt *c*) was purchased from Pharmingen and rabbit polyclonal antibody anti-apoptosis inducing factor (AIF) was purchased from Chemicon Int. 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 [15]; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard [16].

2.3. Standard incubation procedures

Mitochondria (1 mg protein/mL) were incubated in a water-jacketed cell at 20°. 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 given 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 [17].

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

Oxygen uptake was measured by the Clark electrode. 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.

2.5. Detection of Cyt c and AIF release

Mitochondria (1 mg protein/mL) were incubated for 15 min at 20° in the standard medium with the appropriate additions. The reaction mixtures were then centrifuged at 13,000 *g* for 10 min at 4° to obtain mitochondrial pellets. The supernatant fractions were further spun at 100,000 *g* for 15 min at 4° to eliminate mitochondrial membrane fragments and concentrated five times by ultrafiltration through Centrikon 10 membranes (Amicon) at 4°. Aliquots of 10 μL of the concentrated supernatants were subjected to 15% SDS–PAGE for Cyt *c* and 10% SDS–PAGE for AIF and analyzed by Western blotting using mouse anti-Cyt *c* antibody and mouse anti-AIF antibody.

3. Results and discussion

Addition of 10 μM GE to a mitochondrial suspension incubated in standard medium in the presence of 40 μM Ca^{2+} induces a decrease in the apparent absorbance, which is indicative of a large amplitude swelling (Fig. 1A). The inset of Fig. 1A reports the dose-dependent effect of GE on mitochondrial swelling detected after 15 min of incubation (the maximum effect of this compound is obtained at a concentration of 10 μM). The mitochondrial swelling is accompanied by enhanced oxygen uptake (Fig. 1B), followed by a collapse of $\Delta\psi$ (Fig. 1C) and extensive oxidation of endogenous pyridine nucleotides (Fig. 1D). No mitochondrial alteration is observable in control traces in the presence of Ca^{2+} 40 μM alone (dashed lines).

The effects of GE on mitochondria generally are strictly Ca^{2+} dependent. This is demonstrated by the complete inefficacy exhibited by GE in the absence of Ca^{2+} in almost all the parameters evaluated (Fig. 1A, C, and D) except for O_2 uptake (Fig. 1B) or when RLM are treated with EGTA, a Ca^{2+} chelator (data not shown).

All the effects of GE are almost completely blocked by 1 μM cyclosporin A (CsA), that interacts with mitochondrial cyclophyilin, 10 μM bongkrekic acid (BKA), an inhibitor of adenine nucleotide translocase, or 10 μM *N*-ethylmaleimide (NEM), a thiol alkylating agent, all well-known inhibitors of MPT induction [18].

These observations clearly demonstrate that GE, in the presence of supraphysiological Ca^{2+} concentrations is able to induce the transition pore opening.

The induction of MPT represents a catastrophic phenomenon for the organelle and then for the whole cell and has been correlated with the activation of the death machinery of the cell by releasing specific pro-apoptotic mitochondrial proteins, such as Cyt *c* and AIF.

To detect a possible release of these pro-apoptotic proteins from mitochondria induced by GE, RLM were incubated under different conditions for 15 min and then the soluble fraction, separated from the mitochondrial pellet, was subjected to Western blot analysis with two antibodies recognizing Cyt *c* and AIF, respectively. As reported in Fig. 2, the release of Cyt *c* and AIF in the soluble fraction from samples of mitochondrial suspension treated only with 40 μM Ca^{2+}, are nearly undetectable. In contrast, considerable amounts of Cyt *c* and AIF were detected in the supernatants of mitochondria incubated in the presence of 40 μM Ca^{2+} plus 10 μM GE. These releases are completely inhibited if 1 μM CsA, 5 μM BKA, or 10 μM NEM are added to the mitochondrial suspension, indicating that the release of the pro-apoptotic proteins are related to the opening of the membrane transition pore by GE. Loss of Cyt *c* takes place after about 5 min of incubation (Fig. 2B) when considerable swelling and pyridine nucleotide oxidation are also induced (Fig. 1A and D).

The mechanism by which GE induces the MPT is unknown at present. However, on the basis of the observed

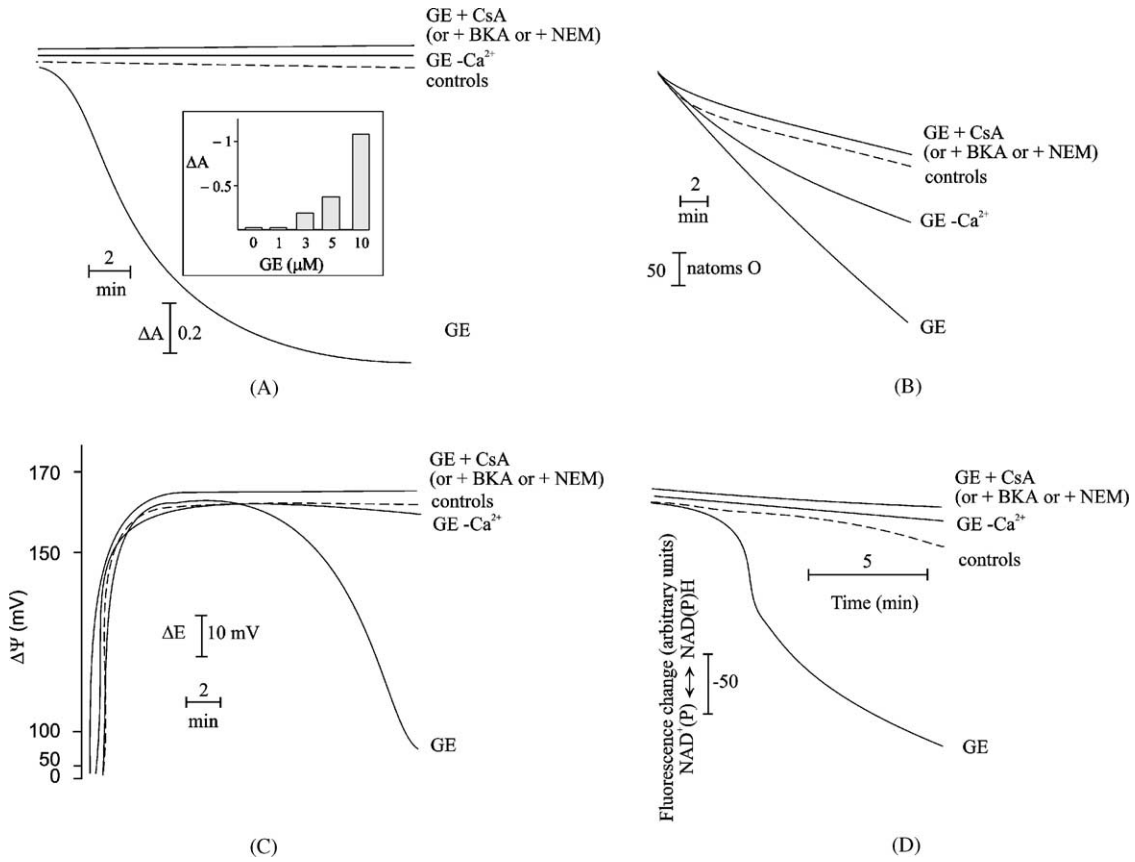

Fig. 1. Mitochondrial swelling (A), oxygen uptake (B), $\Delta\psi$ collapse (C), and pyridine nucleotide oxidation (D) induced by GE in rat liver mitochondria (RLM). RLM were incubated in standard medium supplemented with 40 μM Ca^{2+} under the conditions indicated in Section 2. GE was present at a concentration of 10 μM. When added to the medium: 1 μM CsA, 5 μM BKA, or 10 μM NEM were present. A downward deflection in panels A and C indicates mitochondria swelling and pyridine nucleotide oxidation, respectively. The inset in panel A shows the dose-dependent effect of GE. The ΔE value indicated in panel B refers to the electrode potential. The assays were performed four times with comparable results.

results a suggestion is that GE in the presence of Ca^{2+} is able to induce the transition pore opening by interacting with some pore forming structure. However, the idea that GE alone is able to produce reactive oxygen species (ROS) should not be discarded since it is able to induce a considerable increase in oxygen consumption (Fig. 1B). Pore opening triggers mitochondrial swelling (Fig 1A) which in turn produces ROS by activating the respiratory chain (Fig. 1B) [19]. ROS generation results in pyridine nucleotide oxidation (Fig. 1D) [19], which further amplifies the MPT [20]. When MPT has reached a critical extent (that observable after 5 min as reported in Fig. 2B), Cyt *c* begins to be released, and if the phenomenon is evaluated in the cell it can trigger the pro-apoptotic pathway. The $\Delta\psi$ collapse is most likely concomitant with swelling. The observed delay (Fig. 1C) is ascribable to a reuptake of TPP^+ by a more GE-resistant mitochondrial subpopulation.

It should be pointed out that the concentration of GE able to intensely induce the phenomenon, 10 μM, is close to plasma concentration of GE after licorice ingestion [21]. These results strongly suggest that *in vivo* the observed pro-apoptotic effect by GE [13,14] can be mediated by not only the proposed inhibition of the liver glucocorticoid-metabolizing enzyme 11HSD1 [13,14], but also by MPT induction. Since apoptosis is considered a safety mechanism activated by organisms in order to eliminate abnormal or damaged cells, the two pathways utilized by GE in inducing apoptosis could explain the several beneficial effects exhibited by licorice and its derivatives as anti-inflammatory, antiviral, and anti-carcinogenic agents [22].

The observed anti-carcinogenic effect would result from the potential pro-apoptotic properties proposed for GE, since apoptosis is the aim of cancer therapies [23]. This statement, however, does not explain the antitumor effect, because normal and transformed cells would both be killed by GE. An explanation can be given by taking into account that tumor cells can present particular characteristics that render them more sensitive to the action of the MPT inducers. For example, some cancer mitochondria exhibit an over expression of hexokinase, a protein which facilitates the opening of the transition pore and favors the action of the MPT inducers [24]. Furthermore, considering that tumor mitochondria normally exhibit a very high $\Delta\psi$ [25], an anticancer drug can be facilitated in entering the matrix and to affect some function with the final result of MPT induction [26,27], which potentially can trigger apoptosis.

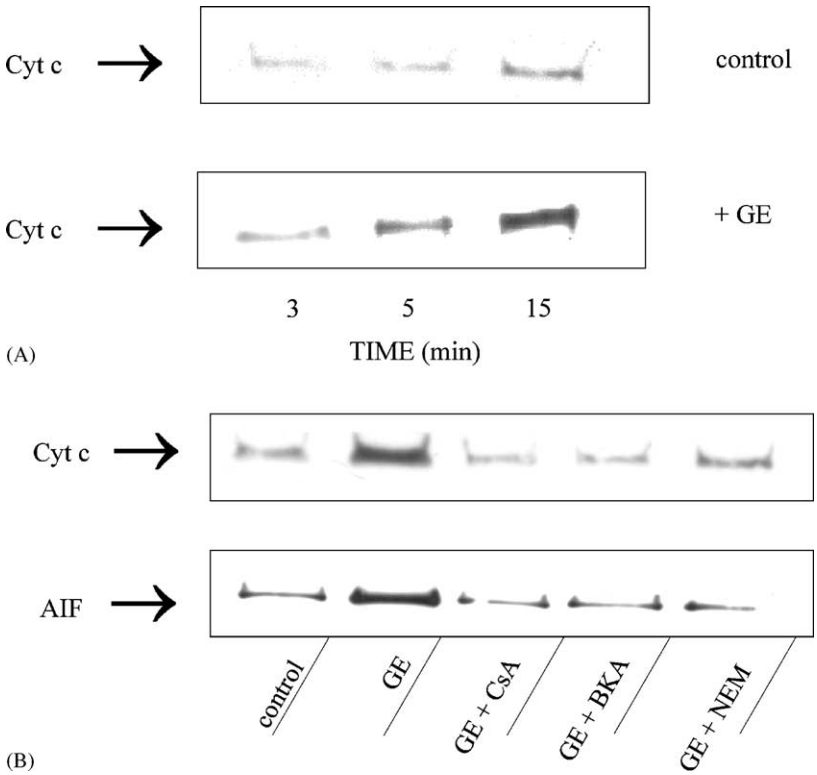

Fig. 2. (A) Time-dependent release of Cyt *c* induced by GE. (B) Release of Cyt *c* and AIF. Inhibitory effect by CsA, BKA, and NEM. Rat liver mitochondria (RLM) were incubated for 15 min in standard medium supplemented with 40 μM Ca^{2+}. Where indicated, 10 μM GE, 1 μM CsA, 5 μM BKA, or 10 μM NEM were present. The incubation was followed by centrifugation and recovery of supernatants that were then concentrated 5-fold, subjected to SDS–PAGE, and immunoblotting to detect Cyt *c* or AIF as described in Section 2. The data shown are typical of three separate experiments.

Acknowledgments

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

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